National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

# ANALYSES OF IMIPRAMINE, PEFLOXACIN AND PEFLOXACIN ANALOGS

BY

**PING SU**

©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**

IN

**PHARMACEUTICAL SCIENCES (DRUG METABOLISM)**

**FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES**

EDMONTON, ALBERTA

**Fall 1993**

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-88049-X

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: **Ping Su**

TITLE OF THESIS: **Analyses of Imipramine, Pefloxacin and Pefloxacin Analogs**

DEGREE: **Doctor of Philosophy**

YEAR THIS DEGREE GRANTED: **Fall 1993**

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

#105, 11110-86Ave, Edmonton, Alberta,

Canada T6G 2P2.

Date: Oct 5. 1993

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **ANALYSES OF IMIPRAMINE, PEFLOXACIN AND PEFLOXACIN ANALOGS** submitted by **Ping Su** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Pharmaceutical Sciences (Drug Metabolism).**

Supervisor R T Coutts
Dr. R.T. Coutts

Supervisor M. Daneshtalab
Dr. M. Daneshtalab

Dr. R.G. Micetich

S E Jensen
Dr. S. Jensen

Glen B Baker
Dr. G.B. Baker

R A Locock
Dr. R.A. Locock

External Examiner RAL
Dr. B. Foster

Date: October 5, 1993

This thesis is dedicated to my mother, Meihua, and father, Yongcheng

"The fear of the Lord is the beginning of wisdom,
and knowledge of the Holy One is understanding."

PROVERBS 9:10

# ABSTRACT

Several commercially available purified human cytochrome P450 (CYP) isozyme preparations provide a unique and reproducible *in vitro* source of the individual CYP isozyme, and make it possible to directly determine the role of the CYP isozyme in a particular metabolic pathway. In this thesis, the utilization of the commercially available CYP isozyme preparations in *in vitro* studies of the metabolism of imipramine, pefloxacin and a newly synthesized pefloxacin analog is described.

An *in vitro* study of the metabolism of imipramine was conducted with the human CYP2D6 isozyme expressed in a human AHH–1 TK+/– cell line to determine the metabolic profile of imipramine with this enzyme preparation. The study clearly shows that CYP2D6 does catalyze the N-demethylation of imipramine to desipramine. 2-Hydroxyimipramine was the major metabolite; a significant amount of desipramine was isolated and 2-hydroxydesipramine was formed in trace amounts. The formation of the secondary metabolite 2-hydroxydesipramine from imipramine can proceed *via* desipramine and *via* 2-hydroxyimipramine, but the former pathway was the preferred one. A combined gas chromatographic/mass spectrometric (GC/MS) analytical procedure with excellent sensitivity was developed and permitted quantification of low levels of imipramine and its metabolites. A procedure involving the O-acetylation of 2-hydroxymetabolites overcame the problem of instability of the metabolites.

A GC analytical procedure was established for analysis of pefloxacin and its metabolites in biological fluids. The novel GC method gave results comparable to those obtained using an HPLC method. An *in vitro* metabolic study of pefloxacin was performed with human CYP2D6, CYP3A4 and CYP1A1 isozymes expressed in a human AHH–1 TK+/– cell line. No metabolite was detected with these enzyme preparations. An *in vivo* metabolic study of pefloxacin was conducted in rat. The major metabolites recovered from rat urine were norfloxacin and an unidentified conjugate.

Pefloxacin analogs, namely pefloxacin quaternary ammonium salts, were designed and synthesized to improve water solubility of this group of compounds. The antibacterial activities of the synthesized compounds were determined. The metabolism of the pefloxacin analogs was studied *in vivo* in the rat and *in vitro* using purified human CYP isozymes.

# ACKNOWLEDGEMENTS

I wish to express my deep gratitude to Dr. Ronald T. Coutts for his enthusiasm, encouragement, support and excellent supervision during the course of this study.

Sincere thanks are extented to Dr. Moshen Daneshtalab and Dr. Ronald G. Micetich for their generous assistance and continued support during the duration of my studies. Special thanks to Dr. Glen Baker, Dr. Sue Jensen, Dr. R. Anthony Locock and Dr. Brian Foster for their helpful suggestions and advice which improve the preparation of this thesis.

Appreciation is also extented to Mr. Don Whyte for his valuble technical assistance, to SynPhar Laboratories Inc. for performing microbiological tests, and to Mr. Joseph Feng (Forest Canada, Edmonton) for providing an access to use HPLC and GC/ECD. Many thanks to Ms. Jane Zhao (Department of Chemistry, University of Alberta) for assistance in the printing of this thesis.

I acknowledge the financial assistance from SynPhar Laboratories Inc., Medical Research Council (MRC) through a grant to Dr.Coutts and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta.

With my deepest apprieciation, I especially thank my parents for their love, encouragement, support and patience.

# TABLE OF CONTENTS

Chapter Page

1 INTRODUCTION ........................................ 1

1.1 METABOLISM ........................................ 2

1.1.1 Cytochrome P450 ........................................ 2

1.1.1.1 Characteristics of P450 ........................................ 2

1.1.1.2 Functions ........................................ 4

1.1.1.3 Distribution ........................................ 6

1.1.1.4 Nomenclature ........................................ 10

1.1.1.5 New systems to study human P450s ........................................ 10

1.1.2 Metabolic N-dealkylation ........................................ 11

1.1.2.1 Mechanism of N-dealkylation ........................................ 12

1.1.2.1 Structure-activity relationships ........................................ 12

1.1.3 *In vitro* and *in vivo* techniques in drug metabolism studies ........................................ 19

1.1.3.1 *In vitro* techniques ........................................ 19

1.1.3.1.1 Purified CYP isozymes ........................................ 22

1.1.3.2 *In vivo* animal techniques ........................................ 24

1.1.3.2.1 Intact animals ........................................ 24

1.2 METHODOLOGY ........................................ 25

1.2.1 Isolation and purification of drugs and their metabolites from biological fluids ........25

1.2.2 Derivatization methods ........26

1.2.3 Gas Chromatography (GC) ........28

1.2.4 High Performance Liquid Chromatography (HPLC) ........28

1.2.5 Mass Spectrometry ........29

1.3 OBJECTIVES OF THE RESEARCH DESCRIBED IN THIS THESIS ........30

1.4 REFERENCES ........31

2 IMIPRAMINE ........45

2.1 INTRODUCTION ........46

2.1.1 Imipramine ........47

2.1.1.1 Chemistry ........47

2.1.1.2 General pharmacology ........49

2.1.1.3 Clinical efficacy ........50

2.1.1.4 Adverse reactions ........51

2.1.1.5 Pharmacokinetics ........52

2.1.2 Metabolism ........52

2.1.3 Analytical methods ........58

2.2 EXPERIMENTAL ........59

2.2.1 Chemicals ........59

2.2.2 Assays ........60

2.2.2.1 Incubation ....................................................................61

2.2.2.2 Work-up without acetylation....................................61

2.2.2.3 Work-up involving acetylation...............................62

2.2.2.4 Instrumental analysis ................................................62

2.2.3 Quantification ...........................................................................63

2.2.3.1 Preparation of calibration curves ............................63

2.2.3.2 Quality control........................................................64

2.2.4 *In vitro* metabolism study.........................................................64

2.2.4.1 Effect of enzyme concentration ...............................64

2.2.4.2 Effect of substrate concentration .............................64

2.2.4.3 Effect of duration of incubation ...............................64

2.2.4.4 Comparison of N-dealkylation and C-hydroxylation pathways ..........................................65

2.2.4.5 Evaluation of CYP3A4 and CYP1A1 activity on N-demethylation of IMI.......................................65

2.3 RESULTS........................................................................................65

2.3.1 Separation of imipramine and its metabolites..........................65

2.3.1.1 Acetylation technique................................................65

2.3.1.2 Technique not utilizing acetylation .........................67

2.3.2 Identification of imipramine and its metabolites.....................67

2.3.2.1 Acetylation technique ...............................................67

2.3.2.2 Technique not utilizing acetylation..........................74

2.3.3 Assays ....82

2.3.4 Metabolism study ....84

2.3.4.1 Effect of enzyme concentration on the yields of metabolites ....84

2.3.4.2 Effect of substrate concentration on the yields of metabolites ....84

2.3.4.3 Effect of duration of incubation on the yields of metabolites ....89

2.3.4.4 Comparison of N-dealkylation and C-hydroxylation pathways ....89

2.3.4.5 Evaluation of CYP3A4 and CYP1A1 activity on N-demethylation of IMI ....91

2.4 DISCUSSION ....91

2.5 REFERENCES ....96

3 PEFLOXACIN ....108

3.1 INTRODUCTION ....109

3.1.1 Pefloxacin ....113

3.1.1.1 Chemistry ....113

3.1.1.2 Microbiology ....113

3.1.1.2.1 Antibacterial activity ....113

3.1.1.2.2 Mode of action ....115

3.1.1.2.3 Development of resistance ....117

3.1.1.3 Pharmacology ....117

3.1.1.4 Clinical trials ....118

3.1.1.4.1 Side effects ........................................................120

3.1.2 Metabolism ........................................................120

3.1.2.1 Drug interactions ........................................................123

3.1.2.1.1 Inhibition of metabolism of methylxanthines by quinolones ........................123

3.1.2.1.2 Inhibition of metabolism of other drugs by quinolones ........................125

3.1.2.2 Identifying the CYP isozyme involved in N-demethylation of pefloxacin ........................126

3.1.3 Analytical method ........................................................127

3.2 EXPERIMENTAL ........................................................130

3.2.1 Materials ........................................................130

3.2.2 Instrumentation ........................................................131

3.2.3 Apparatus ........................................................133

3.2.4 Synthesis of the quinolones ........................................................134

3.2.4.1 Pefloxacin ........................................................134

1-Ethyl-6-fluoro-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (7) ........................134

3.2.4.2 Norfloxacin ........................................................136

1-Ethyl-6-fluoro-1,4-dihydro-7-piperazinyl-4-oxo-3-quinolinecarboxylic acid (8) ........................136

3.2.4.3 Internal standard 1 (ISE) ..........137

1-Ethyl-6-fluoro-7-(4-ethyl-1-piperazine)-4-oxo-3-quinolinecarboxylic acid (9) ..........137

3.2.4.4 Internal standard 2 (ISP) ..........137

1-Ethyl-6-fluoro-1,4-dihydro-7-(4-propyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (10) ..........137

3.2.4.5 Internal standard 3 (ISA) ..........138

1-Ethyl-6-fluoro-7-(4-acetyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (13) ..........138

3.2.5 Derivatization of quinolones ..........139

3.2.5.1 Esterification with methanol ..........139

3.2.5.2 Esterification with 2,2,2-trifluoroethanol (TFE) ..........139

3.2.5.3 Esterification with 2,2,3,3,3-pentafluoropropanol (PFP) ..........141

3.2.6 Analysis of quinolones ..........141

3.2.6.1 Sample preparation ..........141

3.2.6.1.1 Preparation of standard solutions ..........141

3.2.6.1.2 Extraction ..........141

3.2.6.1.3 Derivatization ..........144

3.2.6.1.4 Preparation of calibration curves ..........144

3.2.7 Metabolism studies ..........145

3.2.7.1 Metabolism *in vivo* in the rat ....................................145

3.2.7.2 Metabolism *in vitro* by purified CYP2D6, CYP3A4 and CYP1A1 ....................................146

3.3 RESULTS ....................................147

3.3.1 GC analysis ....................................147

3.3.1.1 Assay ....................................147

3.3.1.2 Metabolic study in the rat ....................................147

3.3.1.3 Metabolic study in purified isozymes (CYP2D6, CYP3A4 or CYP1A1) system ....................................149

3.3.2 HPLC analysis ....................................149

3.3.2.1 Assay ....................................149

3.3.2.2 Metabolic study in the rat ....................................156

3.3.2.3 Metabolic study with purified isozymes (CYP2D6, CYP3A4 or CYP1A1) system ....................................156

3.4 DISCUSSION ....................................158

3.4.1 Derivatization technique ....................................158

3.4.2 Metabolic study in the rat ....................................159

3.4.3 Metabolic study in purified CYP isozymes ....................................163

3.5 REFERENCES ....................................163

4 PEFLOXACIN QUATERNARY AMMONIUM SALTS ....................................175

4.1 INTRODUCTION ....................................176

4.1.1 CNS side effects ....................................177

4.1.2 Structure and biological activity ....................................179

4.1.3 Rationale for designing pefloxacin analogs ........................... 187

4.2 EXPERIMENTAL ........................................................................ 192

4.2.1 Materials ........................................................................ 192

4.2.2 Instrumentation ................................................................ 192

4.2.3 Synthesis of pefloxacin quaternary ammonium salts ............ 193

4.2.3.1 General synthetic procedure ..................................... 193

4.2.3.2 Synthesis of PQAS-1 ................................................. 194

1-Ethyl-6-fluoro-7-(4,4-dimethylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid iodide (PQAS-1) ................................................ 194

4.2.3.3 Synthesis of PQAS-2 ................................................. 194

1-Ethyl-6-fluoro-7-(4-ethyl-4-methylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid bromide (PQAS-2) ............................................ 194

4.2.3.4 Attempted synthesis of PQAS-3 ............................... 195

1-Ethyl-6-fluoro-7-(4,4-diethylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid iodide (PQAS-3) ................................................ 195

4.2.4 Solubility study ................................................................ 195

4.2.4.1 General procedure ..................................................... 195

4.2.5 Microbiology .................................................................... 196

4.2.6 HPLC analysis .................................................................. 196

4.2.6.1 Sample preparation ........ 196

4.2.7 Metabolism studies ........ 196

4.2.7.1 Metabolism *in vivo* in the rat ........ 196

4.2.7.2 Metabolism *in vitro* by purified human CYP isozymes ........ 197

4.3 RESULTS ........ 197

4.3.1 Microbiology ........ 197

4.3.1.1 *In vitro* antibacterial activity ........ 197

4.3.1.2 *In vivo* antibacterial activity ........ 200

4.3.2 Water solubility ........ 200

4.3.3 Metabolism studies ........ 204

4.3.3.1 Metabolism *in vivo* in the rat ........ 204

4.3.3.2 Metabolism *in vitro* by purified human CYP isozymes ........ 204

4.4 DISCUSSION ........ 206

4.4.1 Chemistry ........ 206

4.4.2 Microbiology ........ 206

4.4.3 Metabolism of the quaternary compound ........ 207

4.5 REFERENCES ........ 208

5 CONCLUSIONS ........ 213

# LIST OF TABLES

Table Page

Table 1.1 Some hydroxylation mechanisms in drug metabolism......................5

Table 1.2 Some typical human cytochrome P450 families...............................7

Table 1.3 Metabolic N-dealkylation ..................................................................15

Table 2.1 Accuracy and reproducibility of GC analysis of IMI and its metabolites in enzyme preparation (n=3)............................83

Table 2.2 Metabolic hydroxylation and N-dealkylation of IMI.....................85

Table 2.3 Apparent $K_m$ and $V_{max}$ values of IMI metabolism by CYP2D6..........................................................................................85

Table 3.1 Published HPLC methods for analysis of pefloxacin and its metabolites...............................................................................128

Table 3.2 Charateristics of quinolones derivatized with 2,2,3,3,3-pentafluoropropanol (PFP).................................................142

Table 3.3 Urinary recovery of pefloxacin in the rat .......................................152

Table 3.4 Optimization of derivatization conditions.......................................160

Table 4.1 Some physicochemical properties of quinolones (product information)..........................................................................178

Table 4.2 *In vitro* antibacterial activities of PQAS-1, PQAS-2 and norfloxacin (MIC µg/ml)..........................................................198

Table 4.3 *In vitro* antibacterial activities of PQAS-1, PQAS-2 and norfloxacin against gram-positive bacteria (MIC µg/ml)...................................................................................................199

Table 4.4 *In vivo* antibacterial activity of PQAS-1 (% survival)....................201

Table 4.5 Water solubility of PQAS-1, pefloxacin and norfloxacin (at room temperature).........................................................202

# LIST OF FIGURES

Figure Page

Figure 1.1 A schematic representation of cytochrome P450 ........................3

Figure 1.2 Active site structure of cytochrome P450 that gives rise to the 450 nm maximum absorption in the ferrous carbonyl state ........................3

Figure 1.3 Possible chemical mechanism of the hemoprotein-catalyzed N-demethylation of an N,N-dimethylamine ........................13

Figure 1.4 Structures of some substrates which undergo metabolic N-dealkylation ........................20

Figure 1.5 AHH–1 TK +/– cells were transfected with plasmids containing cytochrome P450 cDNA ........................23

Figure 1.6 Some types of derivatization utilized in GC analyses ........................27

Figure 2.1 Structures of imipramine (IMI) and its metabolites, 2-hydroxyimipramine (2-OH-IMI), desipramine (DMI), 2-hydroxydesipramine (2-OH-DMI), and 10-hydroxyimipramine (10-OH-IMI) ........................48

Figure 2.2 Major pathways of metabolism for imipramine in man ........................54

Figure 2.3 GC traces of a) an acetylated extract of an incubation solution containing authentic samples of IMI, IPR, 2-acetoxy-IMI, N-acetyl-DMI, and 2-acetoxy-N-acetyl-DMI, b) an acetylated extract of an IMI metabolism solution incubated with CYP2D6, c) an acetylated extract of an IMI solution incubated with control microsomes ..... 66

Figure 2.4 GC traces of a) an extract from a control microsomal incubation with IMI (80 μM) and b) an extract from an incubation of CYP2D6 with IMI (80 μM) ..... 68

Figure 2.5 Mass spectra of IMI (peak 1 in Figure 2.3b) and its metabolites (peak 2, 3 and 4 in Figure 2.3b) obtained from an acetylated extract of a solution incubated with CYP2D6 ..... 69

Figure 2.6 Mass fragmentation pathways of a) IMI, b) IPR, c) 2-acetoxy-IMI, d) 2-acetoxy-N-acetyl-DMI and e) N-acetyl-DMI ..... 70

Figure 2.7 Mass spectra of 10-OH-IMI (peak A in Figure 2.9) and its degradation products (peaks B to E in Figure 2.9) ..... 75

Figure 2.8 Common fragmentation ions in the mass spectra of 10-OH-IMI (m.w. 296, peak A in Figure 2.9); 10-acetoxy-IMI (m.w. 338, peak C in Figure 2.9) and 10,11-dehydro-IMI (m.w. 278, peak B in Figure 2.9) ..... 76

Figure 2.9 GC trace obtained during the GC/MS analysis of an authentic sample of 10-OH-IMI which had been subjected to acetylation ..... 77

Figure 2.10 Structures of 10-OH-IMI and its degradation products ..... 78

Figure 2.11 Proposed mass spectral fragmentation pathways of an N-(1-propenyl) analog of 10-OH-IMI (m.w. 251, peak D in Figure 2.9) and its O-acetyl derivative (m.w. 293, peak E in Figure 2.9) formed as decomposition products during GC/MS analysis of acetylated A (in Figure 2.9) ....................79

Figure 2.12 Mass spectra of DMI (peak 2 in Figure 2.4b) and 2-OH-IMI (peak 3 in Figure 2.4b) formed as metabolites of IMI from a CYP2D6 incubation mixture ....................80

Figure 2.13 Mass fragmentation pathways of a) DMI and b) 2-OH-IMI ....................81

Figure 2.14 Dependence of the formation of IMI metabolites on CYP2D6 concentration ....................86

Figure 2.15 Effect of substrate concentration on the yields of IMI's metabolites ....................87

Figure 2.16 Relationship between recovered IMI and its metabolites ....................88

Figure 2.17 Relative efficiencies of metabolic C-hydroxylation (C-OH) and N-demethylation (NDM) ....................90

Figure 3.1 Structures of some fluoroquinolones ....................110

Figure 3.2 Structures of pefloxacin and its four metabolites. ....................112

Figure 3.3 Structure of pefloxacin mesylate dihydrate ....................114

Figure 3.4 The dissociation of pefloxacin ....................114

Figure 3.5 Synthetic routes for pefloxacin (7), norfloxacin (8), ISE (9), ISP (10) and ISA (13) ....................135

Figure 3.6 Derivatizations of quinolones ....................140

Figure 3.7 GC/ECD traces of: a) a derivatized extract of an aqueous solution containing authentic samples of pefloxacin, norfloxacin and internal standard; b) a derivatized extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and c) a derivatized extract of a drug-free rat urine sample....................148

Figure 3.8 a) GC/MS trace of: a) a derivatized extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin, and b) Mass spectra of pefloxacin-PFP and ISP-PFP....................150

Figure 3.9 GC/ECD traces of: a) a derivatized extract of an enzymatically hydrolyzed drug-free rat urine sample; b) a derivatized extract of an enzymatically hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and c) a derivatized extract of an enzymatically hydrolyzed rat urine sample collected from 24-48 h after an *i.p.* dose of pefloxacin....................151

Figure 3.10 GC/NPD traces of: a) authentic sample of pefloxacin-PFP (**17**) and norfloxacin-PFP (**16**); b) a derivatized extract of a pefloxacin solution incubated with CYP3A4; and c) a derivatized extract of a pefloxacin solution incubated with CYP2D6....................153

Figure 3.11 GC/NPD traces of: a) a derivatized extract of a pefloxacin solution incubated with control microsome; and b) a derivatized extract of a pefloxacin solution incubated with CYP1A1....................154

Figure 3.12 HPLC traces of: a) an extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; b) an extract of an alkaline hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; c) an extract of an enzymatically hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and d) an extract of a drug-free urine sample. ......155

Figure 3.13 HPLC traces of: a) an authentic sample of norfloxacin and pefloxacin; b) an extract of a pefloxacin solution incubated with control microsomes; c) an extract of a pefloxacin solution incubated with CYP2D6; d) an extract of a pefloxacin solution incubated with CYP3A4; and e) an extract of a pefloxacin solution incubated with CYP1A1. ......157

Figure 4.1 Structures of some fluoroquinolones that show CNS side effects. ......180

Figure 4.2 Bicyclic heteroaromatic quinolones. ......180

Figure 4.3 Structures of some fluoroquinolones ......183

Figure 4.4 (a) Hofmann elimination of atracurium
(b) Mechanism of Hofmann elimination ......189

Figure 4.5 Metabolic N-demethylation of SA-504 and N-oxidation of the primary metabolite ......190

Figure 4.6 The synthetic routes for fluoroquinolone quaternary ammonium salts. ......191

Figure 4.7 HPLC traces of: a) a rat urine sample collected for 24h after an *i.p.* dose of PQAS-1; b) a rat urine sample collected for 24h after an *i.p.* dose of PQAS-2; and c) a drug-free rat urine sample. ......203

Figure 4.8 HPLC traces of: a) an authentic sample solution of PQAS-1 and pefloxacin; b) PQAS-1 solution incubated with CYP2D6; c) PQAS-1 solution incubated with CYP3A4; d) PQAS-1 solution incubated with CYP1A1; and e) PQAS-1 solution incubated with control microsomes. ....205

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AHH | Aryl hydrocarbon hydroxylase |
| cDNA | Complementary deoxyribonucleic acid |
| CNS | Central nervous system |
| CV | Coefficient of variation |
| CYP | Cytochrom P450 |
| DMI | Desipramine |
| DNA | Deoxyribonucleic acid |
| ECD | Electron capture detector |
| EM | Extensive metabolizer |
| FAB | Fast atomic bombardment |
| FID | Flame ionization detector |
| GC | Gas chromatography |
| GC/MS | Gas chromatography / mass spectrometry |
| GI | Gastrointestinal |
| h | hour |
| HPLC | High performance liquid chromatography |
| 5-HT | 5-Hydroxytryptamine (serotonin) |
| Hz | Hertz |
| IMI | Imipramine |
| IMI.HCl | Imipramine hydrochloride |
| IS | Internal standard |
| *i.p.* | Intraperitoneal |
| IPR | Iprindole |

| | |
|---|---|
| J | Coupling constant |
| kg | Kilograme |
| $K_m$ | Michaelis constant |
| l | litre |
| M | Molar |
| mg | Milligram |
| MIC | Minimum inhibitory concentration |
| min | minute |
| ml | Millilitre |
| m.p. | Melting point |
| MS | Mass spectrometry |
| m.w. | Molecular weight |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Reduced form of $NADP^+$ |
| NE | Norepinephrine |
| nm | nanometer |
| nmol | nanomole |
| NMR | Nuclear magnetic resonance |
| NPD | Nitrogen-phosphorus detector |
| 2-OH-DMI | 2-Hydroxydesipramine |
| 2-OH-IMI | 2-Hydroxyimipramine |
| 10-OH-IMI | 10-Hydroxyimipramine |
| PFP | 2,2,3,3,3-Pentafluoropropanol |
| PM | Poor metabolizer |
| PQAS | Pefloxacin quaternary ammonium salt |

| | |
|---|---|
| $r^2$ | Correlation coefficient |
| TCA | Tricyclic antidepressant |
| TCD | Thermal conductivity detector |
| TFE | 2,2,2-Trifluoroethanol |
| TK | Thymidine kinase |
| UV | Ultraviolet |
| *vs* | Versus |
| $V_{max}$ | Maximal reaction velocity |
| °C | Degree Celsius |
| mg | microgram |
| mM | micromolar |

# Chapter 1. INTRODUCTION

# 1.1 METABOLISM

## 1.1.1 Cytochrome P450

Oxidation is probably the most common reaction in drug metabolism. Most drugs are oxidized by an enzyme system called mixed function oxidase which is composed of a cytochrome P450 isozyme, NADPH-cytochrome P450 reductase and a phospholipid (Hollenberg 1992). This enzyme system exhibits an absolute requirement for NADPH and molecular oxygen for catalytic activity. Cytochrome P450 is the substrate- and oxygen-binding site of the enzyme system, while the reductase serves as an electron carrier, shuttling electrons from NADPH to cytochrome P450, and the phospholipid facilitates the electron transfer process (Williams 1989). Of the three components, cytochrome P450 is important because of its vital role in oxygen activation and substrate binding.

### 1.1.1.1 Characteristics of P450

The cytochrome P450s are composed of two parts, heme (a porphyrin ring into which an iron atom is incorporated) and protein. The porphyrin ring is covalently linked *via* disulfide bridges to the cysteine molecule in the protein (Figure 1.1) (Brock & Madigan 1991). Cytochrome P450 was first identified as a cellular pigment whose reduced form absorbs light strongly at 450 nm when complexed with carbon monoxide (Black & Coon 1987). This unusual spectral property of cytochrome P450s is attributable to the unique ligation of the heme by a cysteinyl thiolate (Figure 1.2) (White & Coon 1980; Black & Coon 1987). Each cytochrome P450 isozyme has an identical heme part but has a different protein structure which is responsible for the different substrate specificities. The purified isozymes exhibit different molecular weights ranging from 46,000 to 58,000 daltons (Black & Coon 1987); they also exhibit different amino acid com-

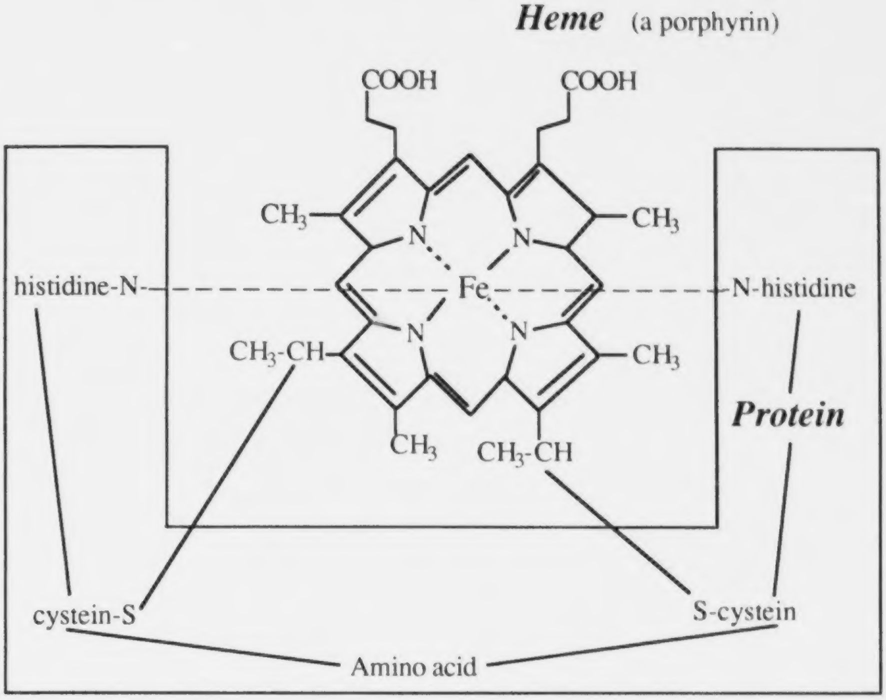

Heme (a porphyrin)
COOH
COOH
CH3
CH3
N
N
Fe
N
N
histidine-N-
-N-histidine
CH3-CH
CH3
CH3
CH3-CH
Protein
cystein-S
S-cystein
Amino acid

Figure 1.1 A schematic representation of cytochrome P450.

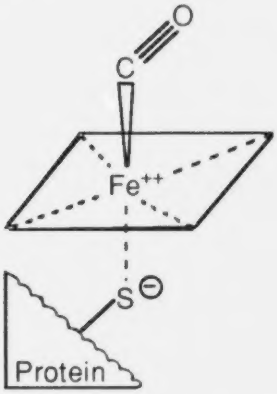

O
C
Fe++
S⊖
Protein

Figure 1.2 Active site structure of cytochrome P450 that gives rise to the 450 nm maximum absorption in the ferrous carbonyl state.

positions, peptide maps, amino acid sequences, and spectral, immunochemical, and catalytic properties.

### 1.1.1.2 Functions

The cytochrome P450 system functions as a multicomponent electron transport system responsible for the metabolism of numerous endogenous compounds such as steroids, bile acids, fatty acids, prostaglandins, leukotrienes, and biogenic amines, as well as for the metabolism of a wide range of xenobiotics including drugs, enviromental pollutants, natural plant products, and alcohols (Nebert *et al.* 1991). The most important function of the cytochrome P450 system is its ability to activate molecular oxygen, catalyzing insertion of one atom of molecular oxygen into the substrate to give initially a monooxygenated product (R-H → R-OH) while the other oxygen atom is reduced to give water (Table 1.1) (Williams 1989). The overall metabolic oxidation catalyzed by P450s proceeds with the stoichiometry shown:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

where RH = substrate; $O_2$ = atmospheric oxygen; NADPH = reduced nicotinamide-adenosine dinucleotide phosphate; and R-OH = oxidised substrate.

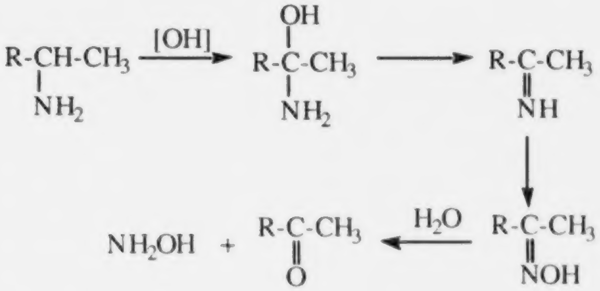
When undergoing P450 catalyzed oxidation, a reactive electrophilic intermediate is generated by incorporation of a hydroxyl group into the hydrophobic substrates. There may be two fates for the electrophilic intermediate *in vivo*: (1) it conjugates with hydrophilic compounds, thereby increasing water solubility and excretion *via* the kidneys, thus leading to detoxification; (2) it may react with the nucleophilic centres of cellular macromolecules initiating toxic damage to the cell. If these nucleophilic centres are located in DNA, DNA

## Table 1.1 Some hydroxylation mechanisms in drug metabolism*

Aromatic hydroxylation: $CH_3\text{-}CO\text{-}NH\text{-}C_6H_5 \xrightarrow{[OH]} CH_3\text{-}CO\text{-}NH\text{-}C_6H_4\text{-}OH$

Aliphatic hydroxylation: $R\text{-}CH_3 \xrightarrow{[OH]} R\text{-}CH_2\text{-}OH$

Deamination:



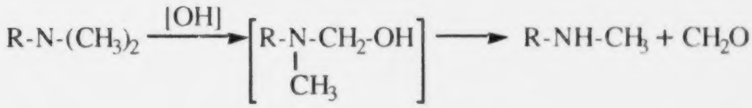
$$R\text{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}\text{-}CH_3 \xrightarrow{[OH]} R\text{-}\overset{\displaystyle OH}{\overset{|}{\underset{\displaystyle NH_2}{\underset{|}{C}}}}\text{-}CH_3 \longrightarrow R\text{-}\underset{\displaystyle NH}{\underset{\|}{C}}\text{-}CH_3$$

$$\big\downarrow$$

$$NH_2OH \; + \; R\text{-}\underset{\displaystyle O}{\underset{\|}{C}}\text{-}CH_3 \xleftarrow{H_2O} R\text{-}\underset{\displaystyle NOH}{\underset{\|}{C}}\text{-}CH_3$$

O-dealkylation: $R\text{-}O\text{-}CH_3 \xrightarrow{[OH]} \left[R\text{-}O\text{-}CH_2\text{-}OH\right] \longrightarrow R\text{-}OH \; + \; CH_2O$

N-dealkylation:



$$R\text{-}N\text{-}(CH_3)_2 \xrightarrow{[OH]} \left[R\text{-}\underset{\displaystyle CH_3}{\underset{|}{N}}\text{-}CH_2\text{-}OH\right] \longrightarrow R\text{-}NH\text{-}CH_3 + CH_2O$$

$R\text{-}NH\text{-}CH_3 \xrightarrow{[OH]} \left[R\text{-}NH\text{-}CH_2\text{-}OH\right] \longrightarrow R\text{-}NH_2 + CH_2O$

S-oxidation: $R\text{-}S\text{-}R' \xrightarrow{[OH]} \left[R\text{-}S\text{-}OH\text{-}R\right] \longrightarrow R\text{-}SO\text{-}R' + H^+$

---

[*] Reference: Williams 1989.

adducts may be formed, causing damage to DNA, resulting in cytotoxicity, mutagenicity and carcinogenicity (Harris 1989).

### 1.1.1.3 Distribution

In mammals the cytochrome P450 system has been detected in almost all tissues examined, including liver, kidney, lung, nasal membrane, brain, intestinal mucosa, bladder, testis, adrenal gland, aorta, and blood platelets. The liver contains the highest concentration of P450, and nasal epithelium the next highest (Black & Coon 1987).

Most of the cytochrome P450 enzymes in eukaryotes (*e.g.* yeast, fungi and mammalian cells) are found in membranes, predominantly in the smooth endoplasmic reticulum and quite often in mitochondria (Guengerich 1992a; Gonzalez 1990). The enzymes from which the P450s receive electrons are different for P450s located in these two intracellular membranes. In the endoplasmic reticulum, NADPH-cytochrome P450 reductase functions as the intermediate electron acceptor, whereas in mitochondria this role is served by two proteins, a ferredoxin-type reductase and a ferredoxin (which contains an iron-sulfur center) (Black 1992).

P450s can be functionally subdivided into two major classes: those involved in synthesis of steroids and bile acids and those that primarily metabolize xenobiotics (Table 1.2) (Gonzalez 1990, 1992). The steroidogenic and cholesterol-metabolizing P450s such as those in families 17, 19 and 21 (discussed below) are highly expressed in extrahepatic tissue and display rather rigid substrate and product specificities. In contrast, xenobiotic-metabolizing P450s, including isozymes in families 1 to 4, are expressed primarily in the liver, but also expressed in extrahepatic tissues to some extent. The xenobiotic-metabolizing

## Table 1.2 Some typical human cytochrome P450 families*

| Class | P450 | Tissues | Inducers** | Substrates |
|---|---|---|---|---|
| Xenobiotic-metabolizing P450s | CYP1A1 | liver, lymphocytes, placenta, lung, colon | smoking, 3-MC, β-NF, ACLR, TCDD | 7-ethoxycoumarin |
| | CYP1A2 | liver | smoking, charbroiled meat, 3-MC, β-NF, ACLR, TCDD | caffeine, arylamines, phenacetin |

---

* References: Gonzalez 1990 & 1992; Murray & Reidy 1990; Nebert *et al.* 1991; Guengerich 1992.

** Abbreviations: β-NF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; ACLR, aroclor 1254; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

**Table 1.2** *(Continued)*

| | | | | |
|---|---|---|---|---|
| | CYP2A6 | liver | | coumarin |
| | CYP2B6 | liver | | cyclophosphamide |
| | CYP2C8, 2C9, 2C10 | liver, intestine | rifampicin, barbiturates | tolbutamide, hexobarbital |
| | CYP2C17, 2C18, 2C19 | liver | | |
| | CYP2D6 | liver, intestine, kidney | | bufuralol, debrisoquine, sparteine |
| | CYP2E1 | liver, intestine, leukocytes | ethanol | ethanol, N-nitrosodialkylamines, acetaminophen |
| | CYP3A3 | liver | | cyclosporin, nifedipine, testosterone |
| | CYP3A4 | liver, gastrointestinal tract | | cyclosporin, nifedipine, testosterone |
| | CYP3A5 | liver, placenta | | |
| | CYP3A7 | liver (fetal) | | |
| | CYP4B1 | lung | | |

**Table 1.2** *(Continued)*

| | | | | |
|---|---|---|---|---|
| Steroidogenic and cholesterol-metabolizing P450s | CYP7 | liver | | cholesterol |
| | CYP17 | steroidogenic tissues | | pregnenolone |
| | CYP19 | steroidogenic tissues | | testosterone,<br>androstenedione |
| | CYP21A2 | steroidogenic tissues | | 17-hydroxy-progesterone |
| | CYP27 | steroidogenic tissues | | cholesterol |

P450s usually exhibit a wide range of, and sometimes overlapping, substrate specificity.

#### 1.1.1.4 Nomenclature

It is now recognized that the cytochrome P450s constitute a multigene family that includes many different isozymes. A nomenclature system for P450 enzymes proposed by Nebert *et al.* (1991) has been widely adopted. The cytochrome P450 superfamily is divided into families, subfamilies and individual enzymes. For naming a P450 gene and corresponding cDNA[1], the nomenclature system contains the italicized root symbol *CYP* denoting *c*y*t*ochrome *P*450, an Arabic number designating the P450 family, a letter indicating the subfamily, and an Arabic numeral representing the individual gene (*e.g. CYP1A2*). For protein in all species, the equivalent nonitalicized symbol is applied (*e.g.* CYP1A2). Those P450 proteins with 40% or greater sequence identity are included in the same family, and those with greater than 55% identity are then included in the same subfamily. There are now 27 known CYP families, of which 10 are predicted to exist in all mammals. These 10 families comprise 18 subfamilies and each subfamily represents a cluster of tightly linked genes (Nebert *et al.* 1991). More than 150 P450 genes have so far been charaterized (Nebert *et al.* 1991), and more than 20 different gene products have been characterized with regard to their properties and catalytic specificities (Guengerich 1992a).

#### 1.1.1.5 New systems to study human P450s

To evaluate the roles of human hepatic P450s in drug metabolism, three levels of investigation must be considered: catalytic activities and substrate specificities of individual P450 forms, the role of individual P450s in the hepatocyte or in subcellular fractions, and finally, metabolism within the whole body.

---

[1] Abbreviation: cDNA, complementary deoxyribonucleic acid.

Traditional approaches useful in determining the role of individual human cytochrome P450s in catalysing a specific oxidation have involved the use of selective inhibitors and antibodies in microsomal preparations, and measurement of catalytic activities using purified enzymes. In recent years, the availability of cDNA-expressed mammalian cytochrome P450s allows direct assessments of structural and functional aspects of various forms of P450. Human P450 cDNAs have been heterologously expressed in a number of systems ranging from standard plasmid transfection to virus-mediated expression of mammalian cells (Gonzalez *et al.* 1991a & 1991b; Porter & Larson 1991; Guengerich *et al.* 1991). Among the most promising systems for study of P450 metabolism is the human B-lymphoblastoid cDNA expression system (Crespi 1991). In this system, cDNA coding for individual human P450 genes (such as *CYP1A2, 2A3, 2D6 or 3A4*) is transfected and expressed in human lymphoblastoid cells (AHH-1 TK+/– cells[2]). Both the microsomal products of individual cell lines and the cell lines themselves have become commercially available (Gentest Corporation). P450 activities can be determined directly by *in vitro* studies.

### 1.1.2 Metabolic N-dealkylation

Oxidative N-dealkylation of secondary and tertiary amines to yield primary and secondary amines, respectively, is one of the most important and most frequently encountered reaction in drug metabolism (Testa & Jenner 1976). Numerous drugs contain an alkylated basic nitrogen atom and many of them undergo metabolic N-dealkylation to a significant and easily detectable extent.

---

[2] AHH, aryl hydrocarbon hydroxylase; TK, thymidine kinase.

### 1.1.2.1 Mechanism of N-dealkylation

The proposed mechanism for oxidative N-dealkylation of, for example, a tertiary amine involves abstraction of an electron from the amine by the cytochrome P450 perferryl oxygen intermediate [$(FeO)^{3+}$ in Figure 1.3], forming an aminium radical cation (I), which then undergoes α-carbon deprotonation to form a neutral carbon-centered radical (II) that recombines with the nascent heme iron-bound hydroxyl radical [$Fe(OH)^{2+}$] to form a carbinolamine (III). The generally unstable carbinolamine would then decompose spontaneously to yield formaldehyde and a secondary amine (Macdonald *et al.* 1989; Hollenberg 1992).

It has been suggested that an N-oxide may serve as an intermediate in the N-demethylation of N,N-dimethylaniline (DMA). To examine this hypothesis, the metabolism of DMA catalyzed by four CYP isozymes (rabbit CYP1A2, 2B4 and rat CYP1A1 and 2B1) was investigated (Hollenberg 1992). No measurable amount of the N-oxide was found. In addition, all four CYP isozymes exhibited greater turnover numbers (two- to seven-fold) for the demethylation of DMA than for the demethylation of DMA N-oxide. These results suggested that the N-oxide is not an intermediate in demethylation catalyzed by these CYP isozymes. Guengerich (1984) has also provided evidence against N-oxides as intermediates in the demethylation of sparteine. It seems that a critical step in these N-demethylations involves initial oxygenation of the α-carbon atom to form a carbinolamine intermediate rather than an N-oxide.

### 1.1.2.1 Structure-activity relationships

An excellent review of oxidative N-dealkylation based on the chemical nature of the substrate has been provided by Testa & Jenner (1976). Substituents that are known to be removed by oxidative N-dealkylation include methyl, ethyl,

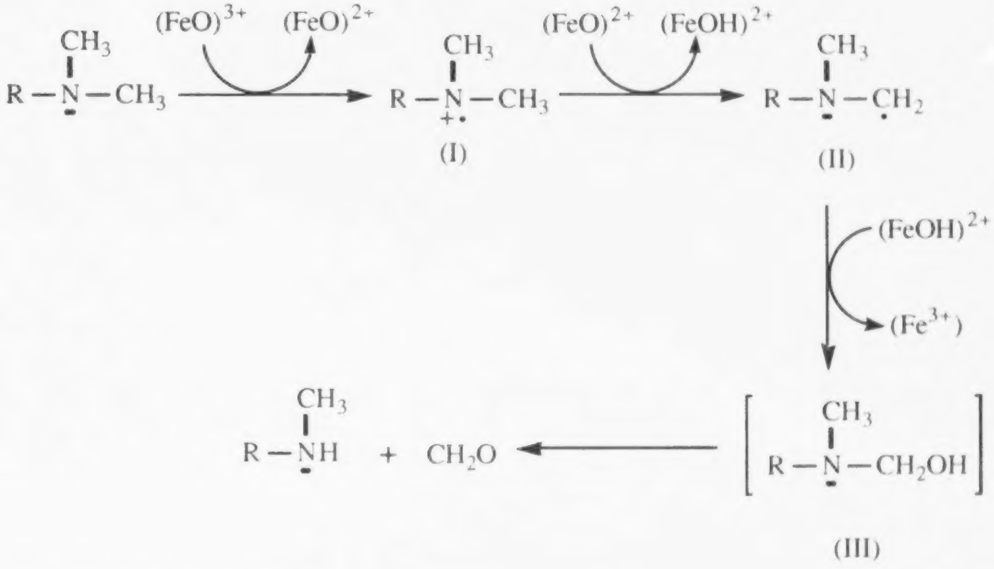

(FeO)$^{3+}$
(FeO)$^{2+}$
(FeO)$^{2+}$
(FeOH)$^{2+}$
(FeOH)$^{2+}$
(Fe$^{3+}$)
R—N—CH$_3$
CH$_3$
(I)
(II)
(III)
R—NH
CH$_2$O
R—N—CH$_2$OH

Figure 1.3 Possible chemical mechanism of the hemoprotein-catalyzed N-demethylation of an N,N-dimethylamine.

n-propyl, isopropyl, n-butyl, allyl, benzyl, and others having an $\alpha$-hydrogen. Substituents that are resistant to N-dealkylation are the tert-butyl (no $\alpha$-hydrogen) and the cyclopropylmethyl groups. Demethylation has also been reported for a few quaternary nitrogen derivatives (Meshi *et al.* 1973). Usually, if two alkyl groups of a different size are attached to the same basic N atom, dealkylation occurs with the smaller alkyl group initially. Dealkylation of tertiary amines to secondary amines generally proceeds at a faster rate than dealkylation of secondary amines to primary amines. This difference in rate has been correlated with lipid solubility (McMahon 1966).

Developments in molecular biology and analytical chemistry have provided much information about cytochrome P450s, both about their regulation and their catalytic specificity (Guengerich 1992b). Several CYP isozymes that are involved in various metabolic N-dealkylations have been identified (Table 1.3). Most of the N-dealkylations surveyed are catalyzed by CYP3A isozymes in human, while 2C, 1A2 and 2D6 isozymes are also involved to some extent. CYP2B1, 2B4, 2C11 and 3A2 isozymes mediate N-dealkylation in various animals (rat, rabbit and sheep). With some substrates, more than one CYP isozyme has been associated with N-dealkylation. One example is the drug propafenone, in which both human CYP3A4 and 1A2 are responsible for its N-dealkylation (Botsch *et al.* 1993). Both rat CYP1A2 and 2C11 isozymes catalyze the N-demethylation of ethylmorphine (Barnett *et al.* 1992; Rane *et al.* 1992). Substrate specificities overlapping species have also been observed. For benzphetamine, the activity of human CYP3A4 overlaps with the activity of sheep or rabbit CYP2B4 (Ged *et al.* 1989; Rodrigues *et al.* 1991; Williams *et al.* 1991), while in codeine N-dealkylation, human CYP3A activity overlaps that of rat CYP2C11

## Table 1.3 Metabolic N-dealkylation

| Drug | Enzyme Source | Isozyme Involved | Remarks | References |
|---|---|---|---|---|
| N-Demethylation | | | | |
| Amiflamine | Human; *in vivo* | CYP2D6 | Both N-mono- and N,N-di-demethylation; note structure is compatible with 2D6 involvement. | Alván *et al.* 1984 |
| Amitriptyline | Human; *in vivo* | Partly CYP2D6 | N-Demethylation induced in smokers so an isozyme other than CYP2D6 is involved. | Mellström *et al.* 1983 and 1986 |
| Antipyrine | Human; *in vivo* | Not CYP2D6 | No correlation with debrisoquine 4-hydroxylation. | Eichelbaum *et al.* 1983<br>Dahlqvist *et al.* 1984 |
| Benzphetamine | Purified rabbit enzyme<br>Sheep lung microsomes<br>Human liver microsomes | CYP2B4<br>CYP2B4<br>CYP3A4 | N-Demethylation inhibited by 3-*t*-butyl-4-hydroxyanisole.<br>Also present in rabbit lung. | Rodrigues *et al.* 1991<br>Williams *et al.* 1991<br>Ged *et al.* 1989 |
| Caffeine | Human liver microsomes | CYP1A2 | N-3-Demethylation is a major pathway. | Butler *et al.* 1989 |
| Citalopram | Human; *in vivo* | A CYP2C isozyme | N-Demethylation decreases in PMs of mephenytoin | Sindrup *et al.* 1993 |
| Clomipramine | Human; *in vivo* | Not CYP2D6 | N-Demethylation strongly inhibited by alcohol abuse. | Balant-Gorgia *et al.* 1987 & 1992 |
| Cocaine | Rat liver microsomes | CYP2B1 | N-Demethylation is phenobarbital-inducible; the product (norcocaine) is further metabolized to a highly toxic nitrosonium ion. | Boelsterli *et al.* 1992<br>Charkoudian *et al.* 1985 |
| | Sheep and rabbit lung microsomes | CYP2B4 | Benzphetamine and N-dimethylnitrosamine are also substrates. | Williams *et al.* 1991 |
| Codeine | Human fetal liver microsomes | A CYP3A isozyme | No O-demethylation [CYP2D6 absent]; Inhibited by midazolam. | Ladona *et al.* 1991 |
| | Rat liver microsomes | CYP2C11 | N-demethylation inhibited by morphine. | Rane *et al.* 1992 |
| Desmethylcitalopram | Human; *in vivo* | CYP2D6 | Didesmethylcitalopram formed in significant amounts in EMs of sparteine but not detectable in PMs. | Gram *et al.* 1993<br>Sindrup *et al.* 1993 |
| Dextro-methorphan | Human fetal liver microsomes | A CYP3A isozyme | No O-demethylation [CYP2D6 absent]. | Ladona *et al.* 1991 |

## Table 1.3 (*Continued*)

| | | | | |
|---|---|---|---|---|
| Diazepam | Human; *in vivo* | Partly CYP2C isozyme but not CYP2D6 | N-Demethylation correlates with S-mephenytoin 4-hydroxylation. | Bertilsson *et al.* 1989 |
| | Rat *in vivo* | Not a CYP2D isozyme | N-Demethylation is phenobarbital-inducible. | Faibushevich *et al.* 1990 |
| | Rat liver microsomes | CYP2B1 | | Jauregui *et al.* 1991. |
| Diltiazem | Human and rabbit liver microsomes | A CYP3A isozyme | Induced by rifampicin, dexamethasone, phenobarbital, phenylbutazone, β-naphthoflavone; inhibited by erythromycin and cyclosporin. | Pichard *et al.* 1990 |
| Dimethylamines (aliphatic and aromatic) | Rabbit liver microsomes | CYP2B1 | N-Demethylation is phenobarbital-inducible. | Burstyn *et al.* 1991 |
| N,N-Dimethylaniline | Rabbit liver microsomes<br>Rat liver microsomes | CYP1A1; CYP2B4<br>CYP1A1; CYP2B1 | Purified rabbit isozyme P450 forms 2 and 4 and rat isozymes P450 b and c catalyze the N-demethylation. | Pandey *et al.* 1989 |
| Dimethylnitrosamine | Pigeon liver microsomes | CYP1A1 | Induced by Aroclor 1254. | Borlakoglu *et al.* 1991 |
| | Sheep lung microsomes | CYP2B4 | Other substrates are cocaine and benzphetamine. | Williams *et al.* 1991 |
| Encainide | Human; *in vivo* | Not CYP2D6 | N-Desmethylencainide formed in significant amounts in PMs of debrisoquine but not detectable in EMs. | Wang *et al.* 1984 |
| Erythromycin and Troleandomycin | Human isozyme expressed in Saccharomyces cerevisiae. | CYP3A4 | CYP3A4 is claimed to be a versatile isozyme. | Brian *et al.* 1990<br>Renaud *et al.* 1990 |
| Ethylmorphine | Rat liver microsomes | CYP1A2 | CYP1A2 levels are increased in hyperinsulinemia. | Barnett *et al.* 1992 |
| | Rat liver microsomes | CYP2C11 | N-Demethylation is inhibited by morphine. | Rane *et al.* 1992 |
| Imipramine | Human; *in vivo* | Not CYP2D6 | Inducible by smoking. | Perel *et al.* 1976 |
| | Human; *in vivo* | Not CYP2D6 | An isozyme other than CYP2D6 is involved | Brøsen *et al.* 1986 |
| | Human; *in vivo* | Partly CYP2D6 | Another CYP isozyme is mainly involved | Brøsen, Gram 1989 |
| | Human liver microsomes | Partly CYP2D6 | Minor reaction at a slow rate. | Skjelbo *et al.* 1991 |
| | Human liver microsomes | Partly CYP2C isozyme | N-Demethylation correlates with S-mephenytoin 4-hydroxylation. | Brøsen *et al.* 1991<br>Skjelbo *et al.* 1991 |
| | Rat liver microsomes | Not CYP2D6 | N-Demethylation strongly inhibited in male rats by antibodies raised to cytochrome P450-m1 (testosterone hydroxylase). | Fujita *et al.* 1989 |
| | Human liver microsomes | Not CYP2D6 or S-mephenytoin hydroxylase | N-Demethylation strongly inhibited by fluvoxamine but not by mephenytoin, citalopram, diazepam, omeprazole or proguanil. | Skjelbo & Brøsen 1992 |

## Table 1.3 (*Continued*)

| | | | | |
|---|---|---|---|---|
| Tamoxifen | Human liver microsomes | A CYP3A isozyme | Strongly correlates with 6β-hydroxylation of testosterone and erythromycin demethylation; inhibited competitively by testosterone, erythromycin, cyclosporin, nifedipine, diltiazem. | Jacolot *et al.* 1991 |
| | Rat liver microsomes | CYP1A, CYP2C and CYP3A isozymes | Results obtained from studies with inducers, inhibitors and antibodies. CYP2A1 is not involved. | Mani *et al.* 1993 |
| Theophylline | Human; *in vivo* | Not CYP2D6 | No correlation with debrisoquine 4-hydroxylation. | Dahlqvist *et al.* 1984 |
| | Human liver microsomes<br>Human; *in vivo* | CYP1A2 | Both N-1- and N-3-demethylation involved.<br>Inhibited by mexiletine. | Sarkar *et al.* 1992<br>Hurwitz *et al.* 1991 |
| Verapamil | Human liver microsomes | CYP3A3/4 | Inhibited by antibodies to CYP3A3/4; CYP2D6 not involved. | Kroemer *et al.* 1991 |
| N-Deethylation | | | | |
| Alkaloid CQA206-291 | Human liver microsomes | CYP3A4 | Inducible by pregnenolone 16α-carbonitrile; potent inhibitors and non-inhibitors described. | Ball *et al.* 1992 |
| Amiodarone | Rat liver microsomes | A CYP3 isozyme | N-Deethylation is inducible by steroids and by phenobarbital. | Rafeiro *et al.* 1990 |
| Lidocaine | Human liver microsomes | CYP3A4 and another CYP isozyme | CYP2D6 is not involved. | Bargetzi *et al.* 1989 |
| | Human liver microsomes<br>Rat liver microsomes | CYP3A4<br>CYP3A2 | Rat CYP3A2 and human CYP3A4 appear to be orthologous. | Imaoka *et al.* 1990 |
| N-De(hydroxyethylation) | | | | |
| Perphenazine | Human; *in vivo* | CYP2D6 | N-dealkylation much reduced in PMs of debrisoquine.<br>Structure does not support involvement of CYP2D6. | Dahl-Puustinen *et al.* 1989<br>Guengerich *et al.* 1986;<br>Meyer *et al.* 1986. |

## Table 1.3 (*Continued*)

| | | | | |
|---|---|---|---|---|
| N-De(dimethoxyphenylethylation) | | | | |
| Verapamil | Human liver microsomes | CYP3A3/4 | Inhibited by antibodies to CYP3A3/4; CYP2D6 not involved; see also N-demethylation. | Kroemer *et al.* 1991 |
| N-Depropylation | | | | |
| Propafenone | Human liver microsomes | Not CYP2D6 | Major metabolic reaction. | Kroemer *et al.* 1989 |
| | Human; *in vivo* | CYP3A4; CYP1A2 | N-Dealkylation is inhibited by verapamil and midazolam. | Botsch *et al.* 1993 |
| N-Desisopropylation | | | | |
| Propranolol | Human; *in vivo* | A CYP2C isozyme | N-dealkylation correlates with mephenytoin 4-hydroxylation. | Ward *et al.* 1989 |
| N-Dealkylation of large side-chain | | | | |
| Alfentanil | Human liver microsomes | CYP3A4 | Formation of noralfentanil correlates with nifedipine oxidation. | Yun *et al.* 1992 |
| Terfenadine | Human liver microsomes | CYP3A4 | C-Hydroxylation of terfenadine is also catalyzed by CYP3A4. | Yun *et al.* 1993 |

(Ladona *et al.* 1991; Rane *et al.* 1992). Examining the substrate specificity of the CYP isozymes involved in N-dealkylation (Table 1.3 & Figure 1.4) reveals that some of the CYP isozymes can tolerate a great deal of diversity in the substrates they can accommodate. CYP3A4, for example, catalyzes oxidative metabolism of a wide range of substrates, which includes high molecular weight drugs such as erythromycin and cyclosporin (Brian *et al.* 1990). Other CYP isozymes such as CYP2B isozymes seem to have at least two active sites, one which accommodates small molecules or a freely rotating nitrogen-containing group [*e.g.* dimethylamine (Burstyn *et al.* 1991)], and the other for bulky molecules which often possess rigid nitrogen moieties [*e.g.* diazepam (Jauregui *et al.* 1991)]. Indeed, understanding the structure-activity relationships that underlie the catalytic specificities of these CYP proteins will be useful in constructing structural models from which the catalytic specificity of human P450s toward new drugs may be predicted.

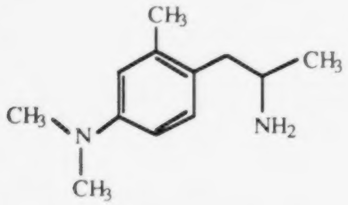
### 1.1.3 *In vitro* and *in vivo* techniques in drug metabolism studies

There are two experimental procedures that are commonly used in drug metabolic studies. One employs *in vitro* conditions, where drug metabolism occurs using an enzyme system isolated from a tissue or organ that has been removed from living body; the other employs *in vivo* where drug metabolism takes place in a natural environment, *i.e.* within an intact living body.

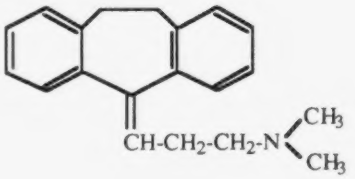
#### 1.1.3.1 *In vitro* techniques

*In vitro* metabolic studies are usually carried out for one or more of the following reasons: 1) To identify in which tissues drug metabolism occurs; 2) To identify primary metabolites and predict possible metabolites formed *in vivo*;

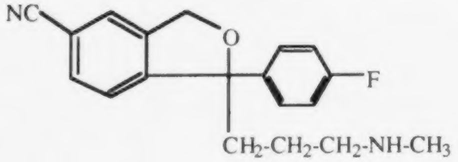
Substrates which undergo N-demethylation catalyzed by CYP2D6:



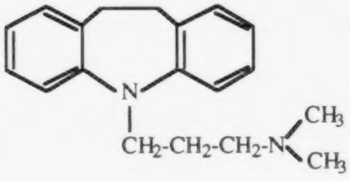
Amiflamine



Amitriptyline

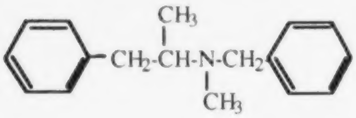


Desmethylcitalopram

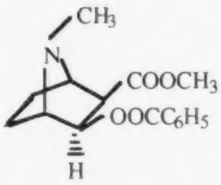


Imipramine

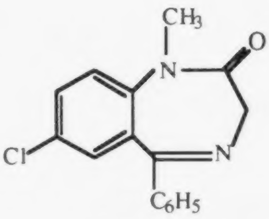
Substrates which undergo N-demethylation catalyzed by CYP2B:



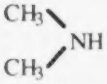
Benzphetamine



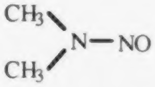
Cocaine



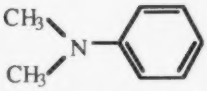
Diazepam



Dimethylamine



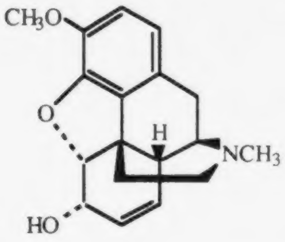
Dimethylnitrosamine



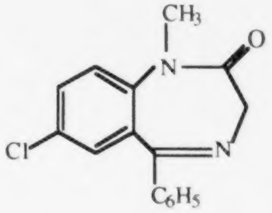
Dimethylaniline

**Figure 1.4 Structures of some substrates which undergo metabolic N-dealkylation.**

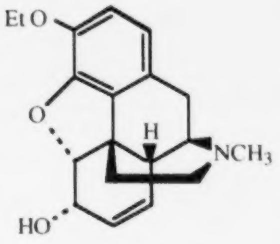
Substrates which undergo N-dealkylation catalyzed by CYP2C:



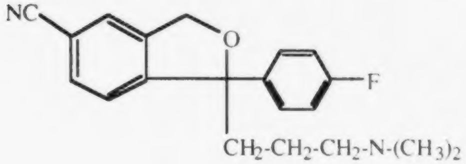
Codeine



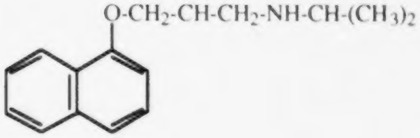
Diazepam



Ethylmorphine

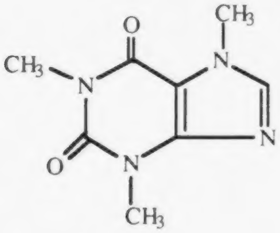


Citalopram

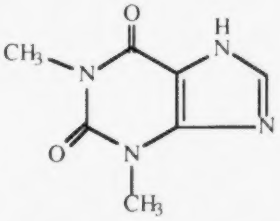


Propranolol

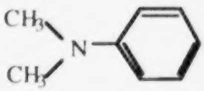
Substrates which undergo N-dealkylation catalyzed by CYP1A2:



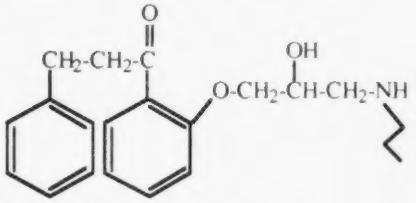
Caffeine



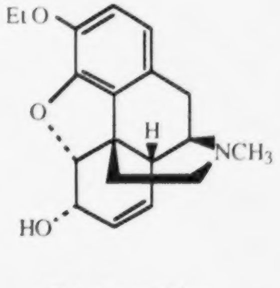
Theophylline



Dimethylaniline



Propafenone



Ethylmorphine

**Figure 1.4** *(Continued)*

3) To elucidate the mechanism(s) of formation of metabolites; 4) To identify the particular enzymes involved in biotransformation. The different preparations used for studies of drug metabolism *in vitro* include isolated organs, tissue slices, isolated cells, subcellular fractions and purified enzymes, especially human cytochrome P450 isozymes (*i.e.* CYP isozymes) expressed in various cell preparations (Gillette 1971; Boobis *et al.* 1990). The *in vitro* technique applied in drug metabolism studies described in this thesis is one which utilizes purified CYP isozymes expressed in human B-lymphoblastoid cell lines.

#### *1.1.3.1.1 Purified CYP isozymes*

Purified enzymes are very useful in the identification of specific isozymes involved in a particular biotransformation. Pure enzymes obtained from cDNA expressed in heterologous systems can be used either as reconstituted systems or as microsomes or as whole cells.

Stable mammalian cell systems that express transfected cytochrome P450s have been developed (Langenbach *et al.* 1992). One of most promising systems is a human B-lymphoblastoid cell line which has been engineered to express a human cytochrome P450 cDNA. The cDNA is isolated primarily from human tissue source and then is inserted into an extrachromosomal plasmid vector (*i.e.* a small, independently replicating genetic element used to replicate genes). The vector contains the Epstein-Barr virus origin of replication (*i.e.* the vector only replicates in Epstein-Barr virus-transformed cells). The plasmid vector into which was incorporated the CYP cDNA of interest is then introduced into a human B-lymphoblastoid cell line (Figure 1.5). This cell line contains Epstein-Barr virus DNA (Gentest 1993).

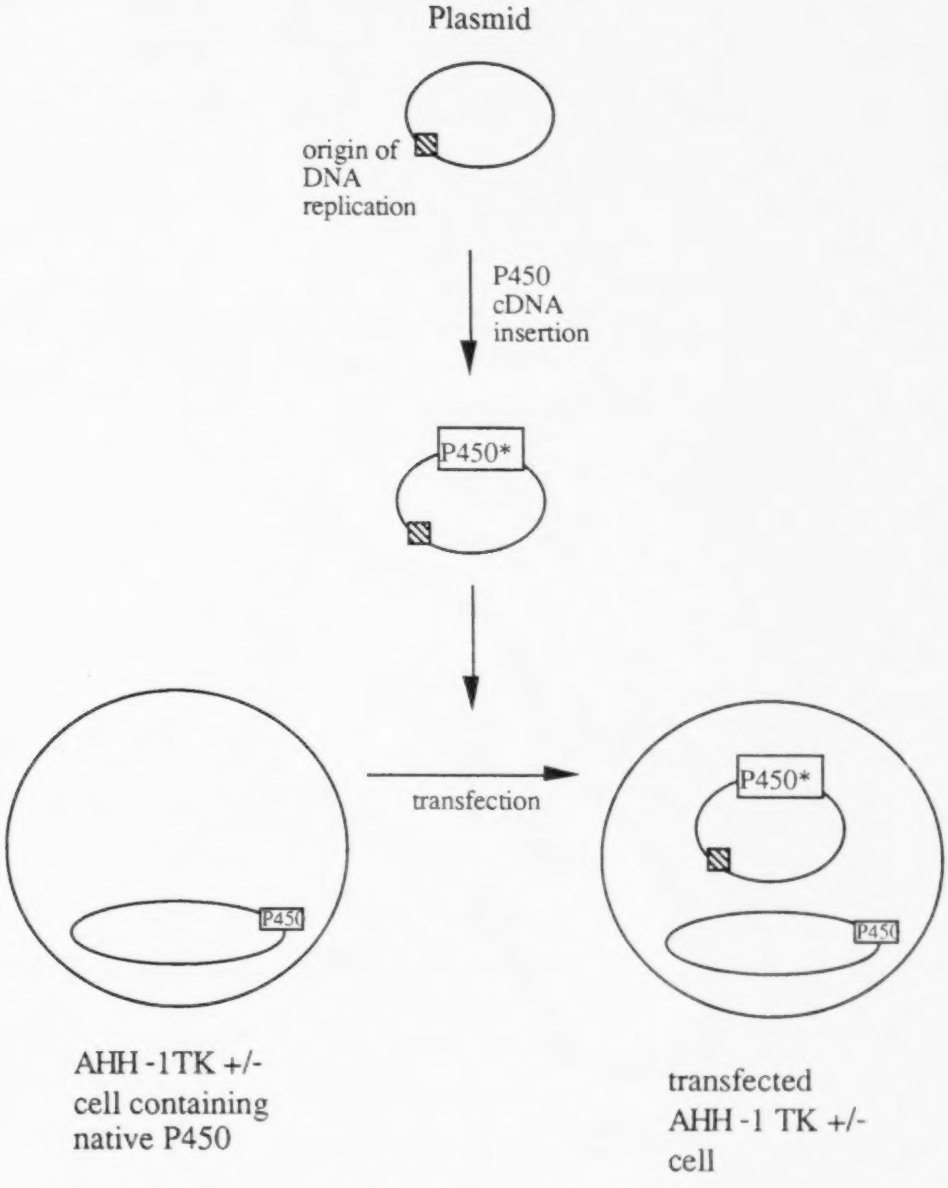

Plasmid
origin of DNA replication
P450 cDNA insertion
P450*
transfection
P450
P450*
P450
AHH -1TK +/- cell containing native P450
transfected AHH -1 TK +/- cell

**Figure 1.5 AHH–1 TK+/– cells were transfected with plasmids containing cytochrome P450 cDNA.**

Microsomal products obtained from the Gentest Corporation are prepared from metabolically competent derivatives of the human AHH-1 TK+/– cell line (one type of human B-lymphoblastoid cell line). The parent AHH-1 TK+/– cell line contains a low level of native human CYP1A1 activity. Microsomes contain the necessary cytochrome P450 reductase/cytochrome $b_5$ and only have to be supplied with appropriate cofactors (NADPH or a NADPH generating system) to be capable of catalyzing oxidative metabolic reactions. The specific activities of cytochrome P450-catalyzed reactions (expressed in pmole product per mg protein per min) in most microsome preparations are within the range of activities observed in human liver microsome preparations.

Metabolic studies utilizing the purified human CYP isozyme require concurrent tests with control microsomes in order to check for the possibility of metabolism by non-P450 enzymes or by low amounts of native CYP1A1 in the microsomes. The control microsomes are prepared from the control cell line which is the AHH-1 TK+/– cell line containing vector without cDNA or containing no vector (untransfected).

### 1.1.3.2 *In vivo* animal techniques

#### *1.1.3.2.1 Intact animals*

Ultimately the information derived from *in vitro* studies must be extrapolated to the intact living animal or human. Male Sprague-Dawley rats were selected as the laboratory animals employed in drug metabolism studies in the present investigations since they are economical, easy to handle and possess enzymatic profiles that are suitable for drug metabolism studies.

## 1.2 METHODOLOGY

### 1.2.1 Isolation and purification of drugs and their metabolites from biological fluids

The extraction of drugs and metabolites from biological samples with water-immiscible organic solvents is the most popular technique used in isolation of basic drugs such as imipramine (IMI). At basic pH the free base can be extracted into a relatively nonpolar organic phase. To remove endogenous impurities efficiently, a three-step method is utilized which usually includes a) the extraction into the organic phase from the aqueous phase at basic pH, b) a reextraction from the organic phase by an acid aqueous phase and c) a repeat extraction of the aqueous phase into organic solvent at basic pH (Gupta 1992). A single basic extraction step into an organic solvent followed by a concentration procedure can be an alternative especially when gas chromatography (GC) is employed (Cooper 1988). Aromatic hydroxy metabolites of basic drugs are more polar than their parent drugs. 2-Hydroxyimipramine (2-OH-IMI) and 2-hydroxydesipramine (2-OH-DMI), for example, are more polar than IMI and desipramine (DMI). Thus, solvents of relatively high polarity such as dichloromethane, diethyl ether or a mixture of ethyl acetate-hexane-isoamyl alcohol (5/4.9/0.1) have been used for extraction (Gupta 1992).

Amphoteric compounds such as pefloxacin having both acidic and basic properties are difficult to extract from an aqueous solution at any pH value. Saturation of the water-diluted biological fluid with inorganic salts (*e.g.* sodium sulfate, ammonium carbonate) is sometimes done before solvent extraction. The main purpose is to obtain effective transfer by 'salting out' the amphoteric analyte into a relatively low volume of organic phase. With this method, the amphoteric drug morphine was recovered in 88-100% yield (Strolin-Benedetti & Caldwell 1990) from an aqueous medium. Solvent extraction is the most commonly applied method in the isolation and purification of fluoroquinolones and their metabolites. Pauliukonis *et al.* (1984) neutralized the norfloxacin-containing plasma or urine sample with phosphate buffer (0.5 M, pH 7.5), and extraction was carried out with dichloromethane. Other solvent systems used include chloroform, a mixture of dichloromethane/isopropanol (7/3 or 9/1) (Dell *et al.* 1988; Vallée *et al.* 1986), and a mixture of chloroform/isopentanol (9/1) (Montay & Tassel 1985). Millipore filters have been directly utilized for purification of serum samples of ciprofloxacin and norfloxacin before HPLC analysis (Nilsson-Ehle 1987).

### 1.2.2 Derivatization methods

GC is a powerful separation technique which has been widely used for analysis of gas, liquid and solid samples. However, not all compounds are accessible to direct analysis by GC, especially those which are polar, have low volatility and/or are thermally unstable. This is the case in drug metabolism studies. Most drug metabolites are more polar than the parent drug. Polar metabolites usually have long GC retention times and produce asymmetric, tailing peaks. Sometimes they fail to elute from the GC column. Derivatization is a technique to improve the GC analysis of these compounds by a) decreasing their polarity, b) increasing their volatility, c) improving their thermal stability, and/or d) introducing specific groups which enhance their detectability (Kline & Soine 1984; Reidman 1973). Derivatization of alcohol, carboxylic or amino groups minimizes the adsorption of these compounds on GC columns (Vandenheuvel & Zacchei 1976; Gupta 1992). The general types of derivatization procedures are esterification, acylation, alkylation, silylation, condensation and cyclization (Figure 1.6) (Kline & Soine 1984; Blau & King 1978). A very useful handbook

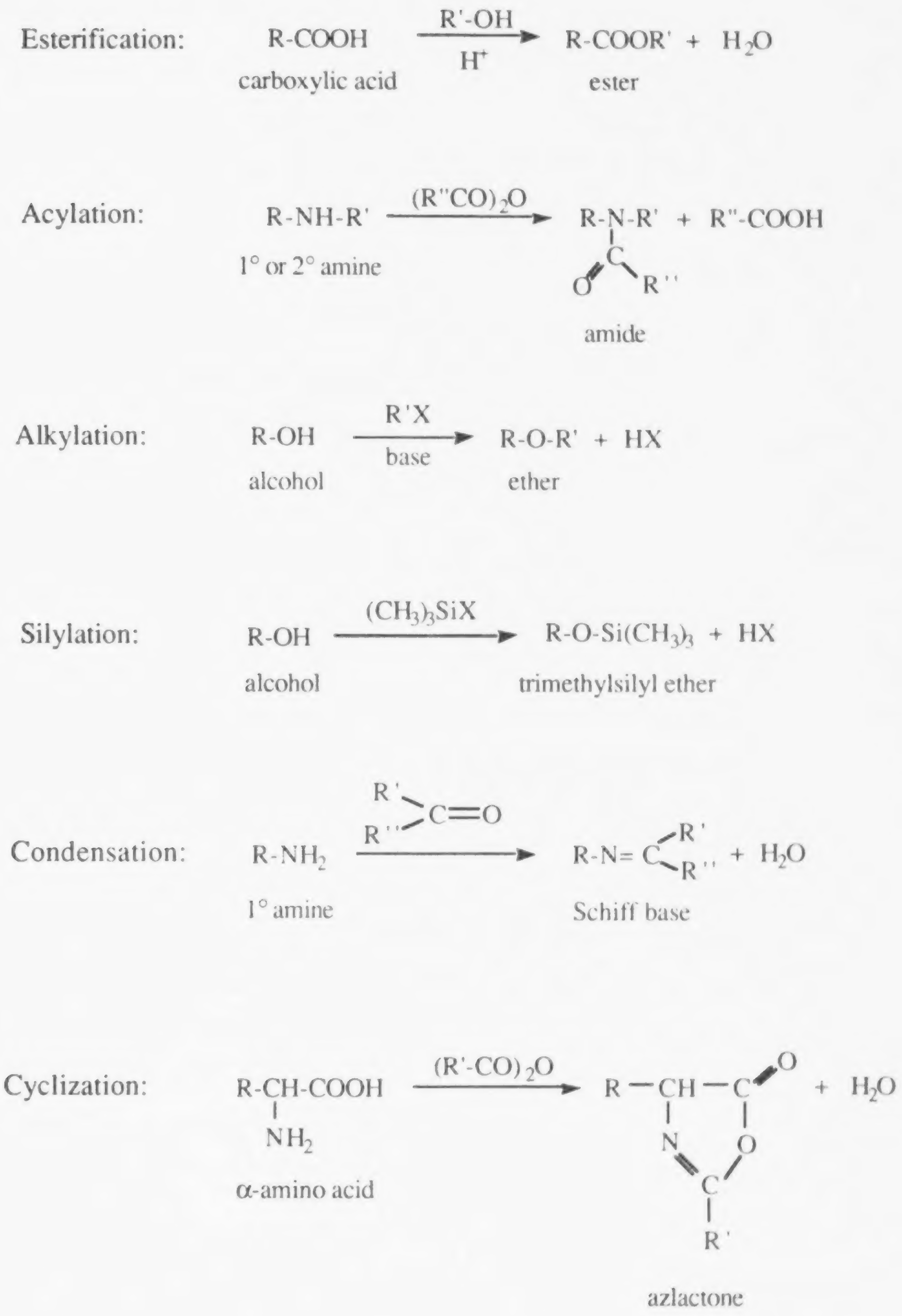

Esterification:
R-COOH
carboxylic acid
R'-OH
H+
R-COOR' + H2O
ester
Acylation:
R-NH-R'
1° or 2° amine
(R"CO)2O
R-N-R' + R"-COOH
C
O
R''
amide
Alkylation:
R-OH
alcohol
R'X
base
R-O-R' + HX
ether
Silylation:
R-OH
alcohol
(CH3)3SiX
R-O-Si(CH3)3 + HX
trimethylsilyl ether
Condensation:
R-NH2
1° amine
R'
R''
C=O
R-N= C
R'
R''
+ H2O
Schiff base
Cyclization:
R-CH-COOH
NH2
α-amino acid
(R'-CO)2O
R — CH — C
O
N
O
C
R'
+ H2O
azlactone

**Figure 1.6 Some types of derivatization utilized in GC analyses.**

on derivatization methods has been provided by Blau and King (1978).

### 1.2.3 Gas Chromatography (GC)

GC methods have been utilized in the analysis of antidepressants (Cooper 1988; Gupta 1992). Both packed and capillary columns have been used, the latter showing much better resolution but at higher cost. Although DB-17 is the most common stationary phase used for the assay of antidepressants, separation of primary amine metabolites from their parent secondary amine drugs is often much better using DB-1 or DB-5 (Gupta 1992).

The selection of an appropriate GC detector depends on the properties of the compound of interest or the properties of its derivatives. The flame ionization detector (FID) and the thermal conductivity detector (TCD) are nonselective whereas the electron capture detector (ECD) and nitrogen-phosphorus detector (NPD) are relatively selective. The NPD is usually the most suitable detector for antidepressants (Gupta 1992).

### 1.2.4 High Performance Liquid Chromatography (HPLC)

The application of HPLC to the separation and quantitation of psychoactive drugs has increased in popularity since the early 1980s. IMI and DMI have been well separated on either normal or reverse phase columns, using UV detection (Carfagnini *et al.* 1990; Segatti *et al.* 1991).

HPLC techniques are used extensively in the analysis of fluoroquinolones, a new group of antibacterial agents (Nilsson-Ehle 1987; Laganá *et al.* 1987; Vallée *et al.* 1986). It has been observed that most of the reported HPLC methods for this class of substances involve the use of ion pair reagents in the mobile phase in order to obtain optimal peak shapes on reversed phase columns.

Tetrabutylammonium salts are commonly used, and heptane sulphonic acid has been identified as a successful alternative (Dell *et al.* 1988).

### 1.2.5 Mass Spectrometry

Mass spectrometry (MS) represents an important technique for identification and characterization of drug metabolites because of its extreme sensitivity and of the highly diagnostic structural information it provides (Rose & Johnson 1982). MS has been immensively useful in metabolic studies, especially when coupled with GC. GC-MS is the most widely used hyphenated technique (the term used to describe the combination of chromatographic methods, which serve to separate complex mixtures into their components, and spectroscopic techniques which provide online characterization of the individual components) (Strolin-Benedetti & Caldwell 1990).

GC-MS has been employed for the analysis and identification of antipsychotic drugs since 1968 (Hammar 1968). IMI and DMI have been measured in plasma and serum by GC-MS (Reed 1988). Derivatization of DMI, 2-OH-IMI and 2-OH-DMI to N-trifluoroacetamides, and the subsquent separation of the derivatives by GC allowed the identification of these compounds by MS (Narasimhachari *et al.* 1981). The high specificity and sensitivity of GC-MS provide the definitive method for characterization of drugs and their metabolites.

## 1.3 OBJECTIVES OF THE RESEARCH DESCRIBED IN THIS THESIS

1. To investigate the metabolism of IMI, pefloxacin and synthesized pefloxacin quaternary ammonium salts *in vitro* with a commercially available human cytochrome P450 isozyme (CYP2D6, CYP3A4 or CYP1A1) preparation. Of particular interest is the involvment of these CYP isozymes in the N-demethylation of these compounds.

2. To develop an analytical protocol for incubation of IMI with fortified enzyme preparation and subsequent sample/product extraction, gas chromatographic separation and GC/MS identification and quantification of the drug and its metabolites.

3. To establish a GC analytical method for quantification of pefloxacin and its metabolites in rat urine. An HPLC analytical method will be compared with the GC method to confirm the validity of the GC method.

4. To synthesize pefloxacin analogs with improved water solubility. The antibacterial activities of the pefloxacin analogs will be determined.

5. To develop an HPLC method for analysis of the synthesized pefloxacin analogs.

6. To study the metabolism of pefloxacin and the synthesized pefloxacin analog *in vivo* in the rat.

## 1.4 REFERENCES

Alván G, Grind M, Graffner C, Sjöqvist F. Relationship of N-demethylation of amiflamine and its metabolite to debrisoquine hydroxylation polymorphism. *Clin Pharm Ther* (1984) **36**: 515-519.

Balant-Gorgia AE, Gay M, Gex-Fabry M, Balant LP. Persistent impairment of clomipramine demethylation in recently detoxified alcoholic patients. *Ther Drug Monit* (1992) **14**: 119-124.

Balant-Gorgia AE, Balant LP, Zysset T. High plasma concentration of desmethylclomipramine after chronic administration of clomipramine to a poor metabolizer. *Eur J Clin Pharmacol* (1987) **32**: 101-102.

Ball SE, Maurer G, Zollinger M, Ladna M, Vickers AEM. Characterization of the cytochrome P-450 gene family responsible for the N-dealkylation of the ergot alkaloid CQA206-291 in humans. *Drug Metab Dispos* (1992) **20**: 56-63.

Bargetzi MJ, Aoyama T, Gonzalez FI, Meyer UA. Lidocaine metabolism in human liver microsomes by cytochrome P450IIIA4. *Clin Pharmacol Ther* (1989) **46**: 521-527.

Barnett CR, Wilson J, Wolf CR, Flatt PR, Ioannides C. Hyperinsulinaemia causes a preferential increase in hepatic P450IA2 activity. *Biochem Pharmacol* (1992) **43**: 1255-1261.

Bertilsson L, Henthorn TK, Sanz E, Tybring G, Säwe J, Villén T. Importance of genetic factors in the regulation of diazepam metabolism: Relationship to S-mephenytoin, but not debrisoquine, hydroxylation phenotype. *Clin Pharmacol Ther* (1989) **45**: 348-355.

Black SD. Membrane topology of the mammalian P450 cytochromes. *FASEB J* (1992) **6**: 680-685.

Black SD, Coon MJ. P-450 cytochromes: structure and function. In: *Advances in Enzymology, 60*. (Meister A ed.), John Wiley & Sons Inc., (1987) pp. 35-87.

Blau K, King GS. In: *Handbook of Derivatives for Chromatography* (Blau K & King GS eds.), Heyden & Son Ltd., London, (1978).

Boelsterli UA, Lanzotti A, Göldlin C, Oertle M. Identification of cytochrome P-450IID1 as the cocaine-bioactivating isoform in rat hepatic microsomes and in cultured rat hepatocytes. *Drug Metab Dispos* (1992) **20**: 96-101.

Boobis AR, Sesardic D, Gooderham NJ. Methods in drug metabolism. In: *Comprehensive Medicinal Chemistry: The Rational Design, Mechanistic Study & Therapeutic Application of Chemical Compounds, Vol 5, Biopharmaceutics*. (Hansch C, Sammes PG & Taylor JB eds.), Pergamon Press plc, Oxford, England, (1990) pp. 443-478.

Borlakoglu JT, Stegeman J, Dils RR. Induction of hepatic cytochrome P-450IA1 in pigeons treated *in vivo* with Aroclor 1254, a commercial mixture of polychlorinated biphenyls (PCBS). *Comp Biochem Physiol* (1991) **99C**: 279-286.

Botsch S, Gautier JC, Beaune P, Eichelbaum M, Kromemer HK. Identification of the cytochrome P450 enzymes involved in N-dealkylation of propafenone: molecular base for interaction potential and variable disposition of active metabolites. *Mol Pharmacol* (1993) **43**: 120-126.

Brain WR, Sari MA, Iwasaki M, Shimada T, Kaminsky LS, Guengerich FP. Catalytic activities of human liver cytochrome P-450IIIA4 expressed in *Saccharomyces cerevisiae*. *Biochemistry* (1990) **29**: 11280-11292.

Brock TD, Madigan MT. Nutrition, metabolism, and biosynthesis. In: *Biology of Microoganisms* Prentice-Hall International, Inc., Englewood Cliffs, New Jersey, (1991) pp. 92-131.

Brøsen K, Zeugin T, Meyer UA. Role of P450IID6, the target of the sparteine-debrisoquine oxidation polymorphism, in the metabolism of imipramine. *Clin Pharmacol Ther* (1991) **49**: 609-617.

Brøsen K, Gram LF. Quinidine inhibits the 2-hydroxylation of imipramine and desipramine but not the demethylation of imipramine. *Eur J Clin Pharmacol* (1989) **37**: 155-160.

Brøsen K, Otton SV, Gram LF. Imipramine demethylation and hydroxylation: impact of the sparteine oxidation phenotype. *Clin Pharmacol Ther* (1986) **40**: 543-549.

Burstyn JN, Iskandar M, Brady JF, Fukuto JM, Cho AK. Comparative studies of N-hydroxylation and N-demethylation by microsomal cytochrome P-450. *Chem Res Toxicol* (1991) **4**: 70-76.

Butler MA, Iwasaki M, Guengerich FP, Kadlubar FF. Human cytochrome P-450A (P-450IA2), the phenacetin O-deethylase, is primarily responsible for the hepatic 3-demethylation of caffeine and N-oxidation of carcinogenic arylamines. *Proc Natl Acad Sci USA*. (1989) **86**: 7696-7700.

Carfagnini G, Corcia AD, Marchetti M, Samperi R. Antidepressants in serum determined by isolation with two on-line sorbent cartridges and liquid chromatography. *J Chromatogr* (1990) **530**: 359-366.

Charkoudian JC, Shuster L. Electrochemistry of norcocaine nitroxide and related compounds: implications for cocaine hepatotoxicity. *Biochem Biophys Res Comm* (1985) **130**: 1044-1051.

Cooper TB. Gas-liquid chromatography of antidepressant, antipsychotic, and benzodiazepine drugs in plasma and tissues. In: *Neuromethods* **10**. (Boulton AA, Baker GB & Coutts RT eds), Humana Press, Clifton, New Jersey, (1988) pp. 65-98.

Crespi CL. Expression of cytochrome P450 cDNAs in human B-lymphoblastoid cells: application to toxicology and metabolite analysis. In: *Cytochrome P450, Methods in Enzymology, **206***. (Waterman MR & Johnson EF eds.), Academic Press Inc., San Diego, (1991) pp. 123-129.

Coon MJ, Ding X, Pernecky SJ, Vaz ADN. Cytochrome P450: progress and predictions. *FASEB J.* (1992) **6**: 669-673.

Dahl-Puustinen ML, Liden A, Alm C, Nordin C, Bertilsson L. Disposition of perphenazine is related to polymorphic debrisoquine hydroxylation in human beings. *Clin Pharmacol Ther* (1989) **46**: 78-81.

Dahlqvist R, Bertilsson L, Birkett DJ, Eichelbaum M, Säwe J, Sjöqvist F. Theophylline metabolism in relation to antipyrine, debrisoquine, and sparteine metabolism. *Clin Pharmacol Ther* (1984) **35**: 815-821.

Dell D, Partos C, Portmann R. The determination of a new trifluorinated quinolone, fleroxacin, its N-demethyl, and N-oxide metabolites in plasma and urine by high performance liquid chromatography with fluorescence detection. *J Liq Chromatogr* (1988) **11**: 1299-1312.

Eichelbaum M, Bertilsson L, Säwe J. Antipyrine metabolism in relation to polymorphic oxidations of sparteine and debrisoquine. *Br J Clin Pharmacol* (1983) **15**: 317-321.

Faibushevich AA, Guliaeva LF, Grishanova AI, Mishin VM, Liakhovich VV. Diazepam metabolism by multiple forms of cytochrome P-450 in rat liver microsomes. *Biokhimiia* (1990) **55**: 1210-1215.

Fujita S, Morimoto R, Chiba M, Kitani K, Suzuki T. Evaluation of the involvement of a male specific cytochrome P450 isozyme in senescence-associated decline of hepatic drug metabolism in male rats. *Biochem Pharmacol* (1989) **38**: 3925-3931.

Ged C, Rouillon JM, Pichard L, Combalbert J, Bressot N, Bories P, Michel H, Beaune P, Maurel P. The increase in urinary excretion of 6β-hydroxycortisol as a marker of human hepatic cytochrome P450IIIA induction. *Br J Clin Pharmacol* (1989) **28**: 373-387.

Gentest Corporation. In: *Human Cytochrome P450 Products and Services.* Gentest Corporation, 6 Henshaw St., Woburn, MA 01801 USA, (1993-1994) p. 2.

Gillette JR. Techniques for studying drug metabolism *in vitro*. In: *Fundamentals of Drug Metabolism and Drug Disposition.* (La Du BN, Mandel HG & Way EL eds), The Williams & Wilkins Company, Baltimore, USA, (1971) pp. 400-418.

Gonzalez FJ. Human cytochromes P450: problems and prospects. *Trends Pharmacol Sci* (1992) **13**: 346-352.

Gonzalez FJ, Aoyama T, Gelboin HV. Expression of mammalian cytochrome P450 using vaccinia virus. In: *Cytochrome P450, Methods in Enzymology, **206***. (Waterman MR & Johnson EF eds.), Academic Press Inc., San Diego, (1991a) pp. 85-92.

Gonzalez FJ, Kimura S, Tamura S, Gelboin HV. Expression of mammalian cytochrome P450 using baculovirus. In: *Cytochrome P450, Methods in Enzymology, **206***. (Waterman MR & Johnson EF eds.), Academic Press Inc., San Diego, (1991b) pp. 93-99.

Gonzalez FJ. Molecular genetics of the P-450 superfamily. *Pharmacol Ther* (1990) **45**: 1-38.

Gram LF, Hansen MGJ, Sindrup SH, Brøsen K, Poulsen JH, Aaes-Jørgensen T, Overø KF. Citalopram, interaction studies with levomepromazine, imipramine, and lithium. *Ther Drug Monit* (1993) **15**: 18-24.

Guengerich FP. Cytochrome P450: advances and prospects. *FASEB J.* (1992a) **6**: 667-668.

Guengerich FP. Characterization of human cytochrome P450 enzymes. *FASEB J.* (1992b) **6**: 745-748.

Guengerich FP, Brian WR, Sari MA, Ross JT. Expression of mamalian cytochrome P450 enzymes using yeast-based vectors. In: *Cytochrome P450, Methods in Enzymology, **206***. (Waterman MR & Johnson EF eds.), Academic Press Inc., San Diego, (1991) pp. 130-145.

Guengerich FP, Distlerath LM, Reilly PEB, Wolff T, Shimada T, Umbenhauer DR, Marti MV. Human-liver cytochromes P-450 involved in polymorphisms of drug oxidation. *Xenobiotica* (1986) **16**: 367-378.

Guengerich FP. Oxidation of sparteines by cytochrome P-450: evidence against the formation of N-oxides. *J Med Chem.* (1984) **27**: 1101-1103.

Gupta RN. Drug level monitoring: antidepressants. *J Chromatogr* (1992) **576**: 183-211.

Hammar CG, Holmstedt B, Ryhage R. Mass fragmentography. Identification of chlorpromazine and its metabolites in human blood by a new method. *Anal Biochem* (1968) **25**: 532-548.

Harris CC. Interindividual variation among humans in carcinogen metabolism, DNA adduct formation and DNA repair. *Carcinogenesis* (1989) **10**: 1563-1566.

Hollenberg PF. Mechanisms of cytochrome P450 and peroxidase-catalyzed xenobiotic metabolism. *FASEB J* (1992) **6**: 686-694.

Hurwitz A, Vacek JL, Botteron GW, Sztern MI, Hughes EM, Jayaraj A. Mexilitene effects on theophylline disposition. *Clin Pharmacol Ther* (1991) **50**: 299-307.

Imaoka S, Enomoto K, Oda Y, Asada A, Fujimori M, Shimada T, Fujita S, Guengerich FP, Funae Y. Lidocaine metabolism by human cytochrome P-450s purified from hepatic microsomes: comparison of those with rat hepatic cytochrome P-450s. *J Pharmacol Exp Ther* (1990) **255**: 1385-1391.

Jacolot F, Simon I, Dreano Y, Beaune P, Riche C, Berthou F. Identification of the cytochrome P450IIIA family as the enzymes involved in the N-demethylation of tamoxifen in human liver microsomes. *Biochem Pharmacol* (1991) **41**: 1911-1919.

Jauregui HO, Ng SF, Gann KL, Waxman DJ. Xenobiotic induction of P-450 PB-4 (IIB1) and P-450c (IAI) and associated monooxygenase activities in primary cultures of adult rat hepatocytes. *Xenobiotica* (1991) **21**: 1091-1106.

Kline BJ, Soine WH. Gas chromatography: theory, instrumentation, and pharmaceutical applications. In: *Pharmaceutical Analysis, Modern Methods, Part A.* (Munson JW ed.), Marcel Dekker Inc., New York, (1984) pp. 1-103.

Kroemer HK, Beaune P, Henderson CJ, Wolf CR, Heidemann H. Identification of cytochrome P-450 isozymes involved in the metabolism of verapamil. *Naunyn-Schmiedebergs Arch Pharmacol* (1991) **343** suppl. R124.

Kroemer HK, Mikus G, Kronbach T, Meyer UA, Eichelbaum M. *In vitro* characterization of the human P-450 involved in polymorphic oxidation of propafenone. *Clin Pharmacol Ther* (1989) **45**: 28-33.

Ladona MG, Lindstrom B, Thyr C, Dun-Ren P, Rane A. Differential foetal development of the O- and N-demethylation of codeine and dextromethorphan in man. *Br J Clin Pharmacol* (1991) **32**: 295-302.

Laganá A, Curini R, D'ascenzo G, Marino A, Rotatori M. High performance liquid chromatographic determination of norfloxacin in human tissues and plasma with fluorescence detection. *J Chromatogr* (1987) **417**: 135-142.

Langenbach R, Smith PB, Crespi C. Recombinant DNA approaches for the development of metabolic systems used in *in vitro* toxicology *Mutation Res* (1992) **277**: 251-275.

Macdonald TL, Gutheim WG, Martin RB, Guengerich FP. Oxidation of substituted N,N-dimethylanilines by cytochrome P-450: estimation of the effective oxidation-reduction potential of cytochrome P-450. *Biochemistry* (1989) **28**: 2071-2077.

Mani C, Gelboin HV, Park SS, Pearce R, Parkinson A, Kupfer D. Metabolism of the antimammary cancer antiestrogen agent tamoxifen I. Cytochrome P-450-catalyzed N-demethylation and 4-hydroxylation. *Drug Metab Dispos* (1993) **21**: 645-656.

McMahon RE. Microsomal dealkylation of drugs, substrate specificity and mechanism. *J Pharm Sci* (1966) **55**: 457-466.

Mellström B, Säwe J, Bertilsson L. Amitriptyline metabolism: Association with debrisoquine hydroxylation in nonsmokers. *Clin Pharmacol Ther* (1986) **39**: 369-371.

Mellström B, Bertilsson L, Lou Y-C, Säwe J, Sjöqvist F. Amitriptyline metabolism: Relationship to polymorphic debrisoquine hydrxylation. *Clin Pharmacol Ther* (1983) **34**: 516-520.

Meshi T, Nakamura S, Kanno T. Metabolic fate of 1,1-dimethyl-5-methoxy-3-(dithien-2-ylmethylene)piperidinium bromide (SA-504). *Chem Pharm Bull* (1973) **21**: 1709-1719.

Meyer UA, Gut J, Kronbach T, Skoda C, Meier UT, Catin T. The molecular mechanisms of two common polymorphisms of drug oxidation — evidence for functional changes in cytochrome P-450 isozymes catalysing bufuralol and mephenytoin oxidation. *Xenobiotica* (1986) **16**: 449-464.

Montay G, Tassel JP. Improved high-performance liquid chromatographic determination of pefloxacin and its metabolite norfloxacin in human plasma and tissue. *J Chromatogr* (1985) **339**: 214-218.

Murray M, Reidy GF. Selectivity in the inhibition of mammalian cytochromes P-450 by chemical agents. *Pharmacol Rev* (1990) **42**: 85-101.

Narasimhachari N, Saady J, Friedel RO. Quantitative mapping of metabolites of imipramine and desipramine in plasma samples by gas chromatographic-mass spectrometry. *Biol Psychiat* (1981) **16**: 937-944.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* (1991) **10**: 1-4.

Nilsson-Ehle I. Assay of ciprofloxacin and norfloxacin in serum and urine by high-performance liquid chromatography. *J Chromatogr* (1987) **416**: 207-211.

Pandey RN, Armstrong AP, Hollenberg PF. Oxidative N-demethylation of N,N dimethylaniline by purified isozymes of cytochrome P-450. *Biochem Pharmacol* (1989) **38**: 2181-2185.

Pauliukonis T, Musson DG, Bayne WF. Quantitation of norfloxacin, a new antibacterial agent in human plasma and urine by ion-pair reverse-phase chromatography. *J Pharm Sci* (1984) **73**: 99-102.

Perel JM, Shostak M, Gann E, Kantor SJ, Glassman AH. Pharmacodynamics of imipramine and clinical outcome in depressed patients. In: *Pharmacokinetics of Psychoactive Drugs: Blood Levels and Clinical Response*. (Gottshalk LA & Merlis S eds.), Spectrum, New York, (1976) pp. 229-241.

Pichard L, Gillet G, Fabre I, Dalet-Beluche I, Bonfils C, Thenot JP, Maurel P. Identification of the rabbit and human cytochromes P-450IIIA as the major enzymes involved in the N-demethylation of diltiazem. *Drug Metab Dispos* (1990) **18**: 711-719.

Porter TD, Larson JR. Expression of mammalian P450 in *Escherichia coli*. In: *Cytochrome P450, Methods in Enzymology, **206***. (Waterman MR & Johnson EF eds.), Academic Press Inc., San Diego, (1991) pp.108-116.

Rafeiro E, Leeder RG, Daniels JM, Brien JF, Massey TE. In vitro hepatic, renal, and pulmonary N-dealkylation of amiodarone. *Biochem Pharmacol* (1990) **39**: 1627-1629.

Rane A, Ask B. A conspicuous down-regulating effect of morphine on essential steroid hydroxylation reactions and certain drug N-demethylations. *J Steroid Biochem Mol Biol* (1992) **41**: 91-98.

Reidman M. Gas chromatographic analysis of drugs and drug metabolites in biological samples. *Xenobiotica* (1973) **3**: 411-434.

Renaud JP, Cullin C, Pompon D, Beaune P, Mansuy D. Expression of human liver cytochrome P450IIIA4 in yeast — a functional model for the hepatic enzyme. *Eur J Biochem* (1990) **194**: 889-896.

Rodrigues AD, Fernandez D, Nosarzewski MA, Pierce WM, Prough RA. Inhibition of hepatic microsomal cytochrome P-450 dependent monooxygenation activity by the antioxidant 3-ter-butyl-4-hydroxyanisole. *Chem Res Toxicol* (1991) **4**: 281-289.

Rose ME, Johnson RAW. In: *Mass Spectrometry for Chemists and Biochemists.* Cambridge University Press, Cambridge, England, (1982).

Sarkar MA, Hunt C, Guzelian PS, Karnes HT. Characterization of human liver cytochromes P450 involved in theophylline metabolism. *Drug Metab Dispos* (1992) **20**: 31-37.

Segatti MP, Nisi G, Grossi F, Mangiarotti M. Rapid and simple high-performance liquid chromatographic determination of tricyclic antidepressants for routine and emergency serum analysis. *J Chromatogr* (1991) **536**: 319-325.

Sindrup SH, Brøsen K, Hansen MGJ, Aaes-Jørfensen T, Overø KF, Gram LF. Pharmacokinetics of citalopram in relation to the sparteine and the mephenytoin oxidation polymorphisms. *Ther Drug Monit* (1993) **15**: 11-17.

Skjelbo E, Brøsen K. Inhibitors of imipramine metabolism by human liver microsomes. *Br J Clin Pharmacol* (1992) **34**: 256-261.

Skjelbo E, Brøsen K, Hallas J, Gram LF. The mephenytoin oxidation polymorphism is partially responsible for the N-demethylation of imipramine. *Clin Pharmacol Ther* (1991) **49**: 18-23.

Strolin-Benedetti M, Caldwell J. Isolation and identification of metabolites. In: *Comprehensive Medicinal Chemistry: The Rational Design, Mechanistic Study & Therapeutic Application of Chemical Compounds.* (Hansch C, Sammes PG & Taylor JB eds.) Pergamon Press plc, Oxford, England, (1990) pp. 479-501.

Testa B, Jenner P. In: *Drug Metabolism, Chemical and Biochemical Aspects.* (Testa B & Jenner P eds.), Mercel Dekker Inc., New York, (1976) pp. 82-97.

Vallée F, LeBel M, Bergeron MG. Determination of ciprofloxacin in biological samples by reversed-phase high performance liquid chromatography. *Ther Drug Monit* (1986) **8**: 340-345.

Vandenheuvel WJA, Zacchei AG. Gas-liquid chromatography in drug analysis. *Adv Chromatogr* (1976) **14**: 199-263.

Wang T, Roden DM, Wolfenden HT, Woosley RL, Wood AJJ, Wilkinson GR. Influence of genetic polymorphism on the metabolism and disposition of encainide in man. *J Pharmacol Exp Ther* (1984) **228**: 605-611.

Ward SA, Walle T, Walle K, Wikinson GR, Branch RA. Propranolol's metabolism is determined by both mephenytoin and debrisoquine hydroxylase activities. *Clin Pharmacol Ther* (1989) **45**: 72-79.

White RE, Coon MJ. Oxygen activation by cytochrome P-450 *Annu Rev Biochem* (1980) **49**: 315-356.

Williams DE, Dutchuk M, Lee MY. Purification and characterization of a microsomal cytochrome P-450 IIB enzyme from sheep lung. *Xenobiotica* (1991) **21**: 979-989.

Williams DA. Drug metabolism. In: *Principles of Medicinal Chemistry 3rd edition*, (Foye WO ed.), Lea & Febiger, Philadelphia, (1989) pp. 79-117.

Yun CH, Okerholm, Guengerich FP. Oxidation of the antihistaminic drug terfenadine in human liver microsomes: Role of cytochrome P-450 3A(4) in N-dealkylation and C-hydroxylation. *Drug Metab Dispos* (1993) **21**: 403-409.

Yun CH, Wood M, Wood AJJ, Guengerich FP. Identification of the harmacogenetic determinations of alfentanil metabolism: cytochrome P-450 3A4. *Anesthesiology* (1992) **77**: 467-474.

# Chapter 2. IMIPRAMINE

## 2.1 INTRODUCTION

Imipramine (IMI), the prototype of tricyclic antidepressants (TCAs) was originally synthesized by the J. R. Geigy, Ltd. Company in Basel, Switzerland in the 1950's as an antihistamine. However, it was through the clinical observations of Kuhn that the antidepressant properties of imipramine hydrochloride (IMI.HCl) were discovered (Kuhn 1957 & 1970). Clinical efficacy permitted IMI to be marketed in Canada and USA in the early 1960's (Hughes 1963; Folsom 1961).

Clinical studies have shown that identical doses of a TCA can result in different plasma steady-state levels in different individuals, and hence different responses to the TCA (Gram & Christiansen 1975; Hammer & Sjoqvist 1967; Nagy & Treiber 1973; Zeidenberg *et al.* 1971). These observations prompted extensive investigations of IMI metabolism in humans and animals with attempts to understand the reasons for the intersubject variability (Rubinstein *et al.* 1983; Spina *et al.* 1984 & 1987; von Bahr *et al.* 1985; Brøsen *et al.* 1986a,b). Excellent reviews on this topic are provided by Glassman & Perel (1973) and Sallee & Pollock (1990). Sources of the individual variability are both genetic and enviromental. The genetic polymorphism observed in the oxidative metabolism of sparteine (Eichelbaum *et al.* 1979) and debrisoquine (Mahgoub *et al.* 1977) is known to involve a particular cytochrome P450 isozyme named CYP2D6, and has an important impact on IMI metabolism. Human *in vivo* studies have indicated that the systemic availability of IMI is increased in individuals who are poor metabolizers of debrisoquine/sparteine. It is of importance to identify the cytochrome P450 isozyme(s) involved in IMI metabolic pathways and thus avoid possible interactions resulting from environmental factors such as smoking and alcohol ingestion, as well as with other drugs, since these factors may influence the function of the isozyme(s) and therefore result in alterations in IMI availability.

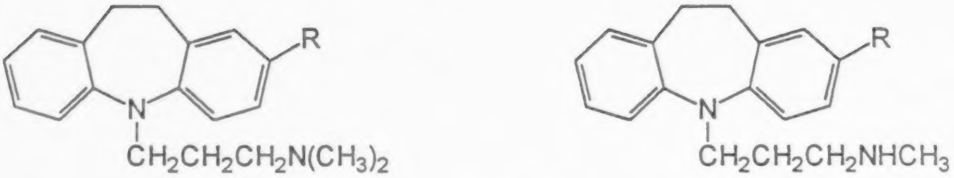
The purpose of the studies to be presented in this chapter is to explore and further define the role of CYP2D6 in IMI metabolism. The major objectives are to examine the involvement of CYP2D6 in various metabolic pathways of IMI, but especially in metabolic N-demethylation, and to investigate the utility of purified CYP2D6 expressed in a human AHH–1 TK+/– cell line in drug metabolism studies with IMI.

### 2.1.1 Imipramine

#### 2.1.1.1 Chemistry

In early 1950's, chemists at J. R. Geigy in Basel, Switzerland, synthesized a group of iminodibenzyl derivatives as potential antihistaminic agents (Schindler & Hafliger 1954). Pharmacologic testing of these compounds revealed that some compounds possessed hypnotic and analgesic properties in addition to their antihistaminic and atropine-like actions (Grünthal 1958). Meanwhile, the demonstration of the antipsychotic effects of the phenothiazines had also stimulated interest in the iminodibenzyl derivatives because of their structural similarity to the phenothiazines and because they shared some common pharmacologic properties, in particular a sedative effect (Klerman & Cole 1965).

For these reasons, one compound of the group (10,11-dihydro-N,N-dimethyl-5H-dibenz[b,f]azepine-5-propanamine), now known as imipramine (Figure 2.1), was evaluated in chronic schizophrenic and other psychotic patients. Following his careful observations, Kuhn (1957) reported that IMI had an antidepressive action. Over the last 30 years, IMI has proved to be one of the most effective agents among the various drugs used for the treatment of

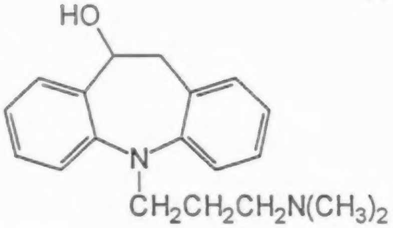

R
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
R
N
CH$_2$CH$_2$CH$_2$NHCH$_3$

Imipramine (IMI), R = H

2-Hydroxyimipramine (2-OH-IMI), R = OH

Desipramine (DMI), R = H

2-Hydroxydesipramine (2-OH-DMI), R = OH


HO
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$

10-Hydroxyimipramine (10-OH-IMI)

**Figure 2.1** **Structures of imipramine (IMI) and its metabolites, 2-hydroxyimipramine (2-OH-IMI), desipramine (DMI), 2-hydroxydesipramine (2-OH-DMI), and 10-hydroxyimipramine (10-OH-IMI).**

depression. IMI and its metabolite desipramine (DMI) continue to be among the most efficacious antidepressants available for the treatment of depression.

#### 2.1.1.2 General pharmacology

The biogenic amine deficiency theory of depression implies that a state of depression is caused by a functional deficiency of norepinephrine (NE) and/or serotonin (5-hydroxytryptamine, 5-HT) at synapses in brain. Therefore, if presynaptic reuptake of transmitter (NE or 5-HT, or both) were inhibited, TCAs could increase neurotransmitter concentrations to normal synaptic levels and thereby alleviate depression (Horwell 1985). Mono-N-demethylated TCAs such as DMI are secondary amines and are most effective in blocking NE reuptake, while those that retain their N,N-dimethyl group, such as IMI, are tertiary amines and are more potent in blocking the reuptake of 5-HT. However, this theory cannot explain observed differences in potency among antidepressants and the relative selectivity of different TCAs in the inhibition of neuronal reuptake of NE and 5-HT. In fact, some effective antidepressants such as iprindole neither inhibit reuptake nor inhibit metabolism of neurotransmitters (Stahl & Palazidou 1986). Also, some drugs such as cocaine block amine reuptake but are not effective antidepressants. The neurotransmitter deficiency theory also fails to explain why antidepressants must be administered for 2 to 3 weeks before they are effective, whereas blockade of neuronal reuptake occurs soon after drug treatment begins. The exact mechanism of action of TCAs thus is still not clear.

Other theories such as neurotransmitter receptor dysregulation hypotheses have also been proposed to explain the onset of depression (Bakish 1991). Such hypotheses propose that dysregulation of the sensitivity of the adrenergic postsynaptic receptors to NE and serotoninergic postsynaptic receptors to 5-HT cause depression. Postsynaptic receptors participate in nerve impulse neurotransmission while the presynaptic receptors regulate neurotransmitter release, reuptake and synthesis. Antidepressant treatment produces complex changes in the sensitivities of both presynaptic and postsynaptic receptor sites (Baker & Greenshaw 1989). The net effect is thought to be the re-regulation of an abnormal receptor-neurotransmitter relationship, although much still remains unknown about the mechanisms of action of antidepressants.

In addition to its antidepressant effect, IMI also produces sedation, anticholinergic effects, and mild peripheral vasodilator effects. The sedative effect of IMI is thought to be related to blockade of α-1-adrenoceptors and histamine receptors (Svensson 1984). IMI exerts prominent effects on the autonomic nervous system. Its blockade of muscarinic cholinergic receptors results in atropine-like effects such as dry mouth, constipation, blurring of vision and urinary retention. At therapeutic doses, IMI produces cardiovascular effects (Muller *et al.* 1961) such as postural hypotension, tachycardia and cardiac arrhythmia. These effects may be attributed to the increased concentrations of NE in cardiac tissue that result from the blockade of transmitter reuptake and to the anticholinergic activity of the drug.

### 2.1.1.3 Clinical efficacy

IMI is one of the most widely prescribed drugs for the treatment of depression. It can be used alone or in combination with neuroleptics and antianxiety agents. In depressed patients, the antidepressant activity usually appears after 14-21 days of multiple daily dosing, although the major side effects appear within a few days (Klerman & Cole 1965). For antidepressant treatment, the recommended therapeutic plasma concentration range (IMI plus DMI) is 240-500 μg/L (Gram *et al.* 1984).

The efficacy of IMI in the treatment of nocturnal enuresis in children is also well established (Blackwell & Currah 1973). An effective plasma level for this effect was found to be 60-80 μg/L (IMI+DMI), much lower than the concentration required for antidepressant effects (Gram *et al.* 1982). Treatment of pain in diabetic neuropathy also requires a lower plasma concentration of IMI (about 80-100 μg/L) to be effective (Kvinesdal *et al.* 1984). It has been suggested that IMI can be used for the treatment of cardiac arrhythmias, and for this purpose effective plasma concentrations were reported to be in the 150-250 μg/L range (Bigger *et al.* 1977). IMI has also been reported to be potentially useful in the treatment of panic attacks and agoraphobia (Mattick 1990) and anxiety disorders (Lydiard 1988).

#### 2.1.1.4 Adverse reactions

The most common side effects of IMI are direct extensions of its pharmacological actions. IMI frequently causes hypotension, particularly orthostatic hypotension with associated vertigo, tachycardia and electrocardiographic (ECG) abnormalities (Krogh 1992). Other cardiac effects less frequently observed include congestive heart failure and myocardial infarction (Kristiansen 1961; Sloman 1960). Thus, great care must be exercised especially when IMI is prescribed to elderly patients, patients with cardiac impairment or when the treatment requires higher dosage than usual. The typical anticholinergic effects mentioned before may become serious problems in elderly patients. An inflammation of the parotid gland may be provoked in the dehydrated patient, and gastrointestinal (GI) effects may produce fecal impaction and paralytic ileus.

#### 2.1.1.5 Pharmacokinetics

When given orally, IMI is well absorbed (> 95%) from the GI tract, with peak plasma levels being reached in 2 to 5 hours ($t_{max}$) and with a plasma half-life ($t_{1/2}$) ranging from 9 to 20 hours (Krogh 1992). The complete absorption of the oral dose was demonstrated by the similar total urinary recoveries (about 45%) obtained after oral and intramuscular dosing (Sutfin *et al.* 1984). IMI is a highly tissue bound drug with an apparent volume of distribution ($V_d$) in the range of 10 to 20 L/kg. Plasma protein binding of IMI is approximately 80%, ranging from 60 to 96% (Sallee & Pollock 1990). This is not surprising for a basic drug such as IMI since lipophilicity of basic drugs has been shown to be a significant determinant of plasma protein binding (Dayton *et al.* 1980). IMI undergoes extensive first-pass metabolism (Gram & Christiansen 1975). Less than 1.5% of an oral dose is recovered as intact drug in urine. Thus the bioavailability of IMI is less than complete and its systemic availability is reported to range from 22 to 50% (Sutfin *et al.* 1984). It has been shown that the sparteine oxidation phenotype has a significant impact on first-pass metabolism. In poor metabolizers (PMs), systemic availability of IMI is about 2-fold greater than in extensive metabolizers (EMs) (Brøsen & Gram 1988) due to the absence of sparteine oxygenase (CYP2D6) in PMs.

### 2.1.2 Metabolism

The metabolism of IMI has been extensively studied, and excellent reviews are available (Glassman & Perel 1973; Gram & Christiansen 1975; Judd & Ursillo 1975; Sallee & Pollock 1990). A scheme showing the major pathways of metabolism of IMI in man is given in Figure 2.2. In addition, the metabolism has also been studied in mouse, rabbit, rat and microorganisms (Herrmann & Pulver 1960; Aeppli 1969; Bickel & Minder 1970; Hufford *et al.* 1981).

In humans, IMI is metabolized *via* two major pathways: N-demethylation to DMI and 2-hydroxylation to 2-hydroxyimipramine (2-OH-IMI). DMI is further metabolized to 2-hydroxydesipramine (2-OH-DMI) *via* 2-hydroxylation (Sutfin *et al.* 1984; Crammer *et al.* 1969). All three major metabolites are pharmacologically active. Minor pathways of metabolism for IMI include iminodibenzyl formation (dealkylation of the entire side chain), IMI-N-oxide formation, 10-hydroxylation of IMI and DMI to 10-hydroxyimipramine (10-OH-IMI) and 10-hydroxydesipramine (10-OH-DMI), respectively, and further demethylation of DMI to didemethylimipramine (DDMI) (Weder & Bickel 1968; Crammer *et al.* 1969; Potter & Calil 1981).

Both IMI and DMI are excreted primarily as their 2-hydroxy metabolites. The hydroxylated metabolites are excreted either in the urine (60-80%) or *via* the biliary system (22%) mainly as glucuronide conjugated forms (Judd & Ursillo 1975). Since both IMI and DMI are excreted as free and conjugated hydroxy metabolites in urine, renal clearance determines their steady-state concentration. In patients suffering from chronic renal failure, increased 2-hydroxy metabolite plasma levels have been demonstrated (Lieberman *et al.* 1985). Kitanaka *et al.* (1982) observed a decrease in renal clearance of 2-OH-DMI in elderly patients. Recently, a positive correlation between age and 2-OH-DMI concentration was demonstrated in patients over 60 (Nelson *et al.* 1988). Thus it is clear that the kinetics of hydroxylated metabolites would be influenced particularly by renal function. 2-OH-IMI and 2-OH-DMI possess comparable activity to IMI and DMI in blocking the reuptake of 5-HT and NE, respectively (Potter *et al.* 1984).

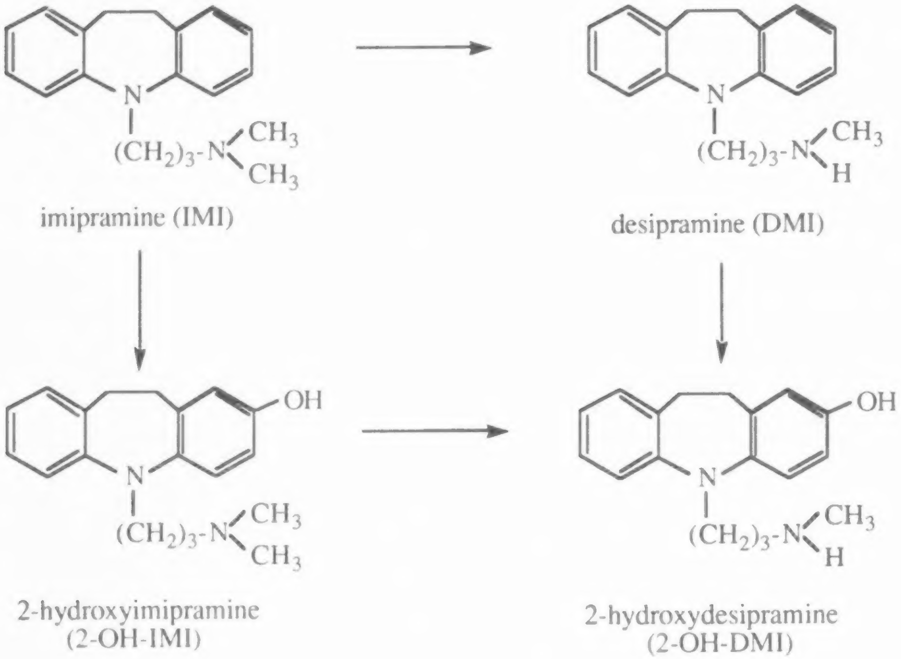

N
(CH2)3-N
CH3
CH3
imipramine (IMI)
N
(CH2)3-N
CH3
H
desipramine (DMI)
OH
N
(CH2)3-N
CH3
CH3
2-hydroxyimipramine
(2-OH-IMI)
OH
N
(CH2)3-N
CH3
H
2-hydroxydesipramine
(2-OH-DMI)

**Figure 2.2 Major pathways of metabolism for imipramine in man.**

IMI has been shown to be metabolized in liver, as demonstrated by *in vitro* experiments with human liver microsomal enzyme preparations (Zeugin *et al.* 1990; Brøsen *et al.* 1991). Extrahepatic tissues such as lung, kidney and gut are essentially not involved in metabolism of IMI in human (Minder *et al.* 1971; Dencker *et al.* 1975). It has been shown that the N-demethylation of IMI and ring 2-hydroxylation of IMI are catalyzed by at least two different isozymes of cyctochrome P450 (Brøsen *et al.* 1986a & b; Brøsen & Gram 1989; Skjelbo *et al.* 1991).

Human *in vivo* studies on the relationship between IMI and DMI metabolism and sparteine-debrisoquine oxidation polymorphism strongly suggested that the 2-hydroxylation of IMI and DMI and 4-hydroxylation of debrisoquine are under control of the same enzyme, which has been identified as the CYP2D6 isozyme (Spina *et al.* 1984; Brøsen *et al.* 1986a; Steiner *et al.* 1987; Brøsen & Gram 1989). Spina *et al.* (1984) observed a strong correlation ($r = 0.92$) between the urinary ratio DMI/2-OH-DMI and that of debrisoquine to 4-hydroxydebrisoquine in 18 healthy subjects who had been phenotyped as EMs or PMs of debrisoquine. Pretreatment with quinidine, a potent inhibitor of CYP2D6, reduced the urinary recovery of 2-OH-DMI 30-fold in EMs but only 3-fold in PMs (Steiner *et al.* 1987). By giving single oral doses of 100 mg IMI and DMI to 18 healthy volunteers (12 EM and 6 PM of sparteine), Brøsen *et al.* (1986b) found that 2-OH-IMI and 2-OH-DMI were detectable only in the plasma of the 12 EMs, but not in the plasma of 6 PMs, and DMI was detectable in the plasma of both EMs and PMs with longer elimination half lives in PMs than in EMs. This finding suggested that 2-hydroxylation of IMI and DMI is catalyzed almost exclusively by sparteine/debrisoquine oxygenase while the N-demethylation of IMI is dependent on different isozyme(s).

Similar results were obtained in human *in vitro* studies (von Bahr *et al.* 1985; Brøsen *et al.* 1991; Birgerssion *et al.* 1986). von Bahr *et al.* (1985) demonstrated that drugs known to be metabolized by debrisoquine hydroxylase competitively inhibited DMI 2-hydroxylation. Brøsen *et al.* (1991) investigated the metabolism of IMI by human liver microsomes from EMs and PMs and found that the formation of 2-OH-IMI was 8-fold higher in EMs than in PMs. Other metabolites of IMI detected in this *in vitro* system were DMI as a major metabolite and 10-OH-IMI in a very small quantity. It was also shown by means of inhibition studies with quinidine and levomepromazine, and by using antibodies against CYP2D6, that this isozyme was not implicated in the conversions of IMI to DMI and 10-OH-IMI. Surprisingly, unlike the human *in vivo* results where 2-OH-DMI was one of the major metabolites when IMI was administered orally, no 2-OH-DMI was isolated in the *in vitro* study. Using the same HPLC analytical method, Zeugin *et al.* (1990) obtained similar results in a metabolism study of IMI by human liver microsomes.

Both human *in vivo* and *in vitro* studies indicated that the 2-hydroxylation of IMI and DMI, but not the demethylation of IMI, may correlate with the sparteine/debrisoquine oxidation polymorphism. However, direct *in vitro* evidence with purified CYP2D6 in support of these findings is very limited. Birgersson *et al.* (1986) isolated and purified a human cytochrome P450 isozyme that was capable of 4-hydroxylating debrisoquine and therefore appeared to be CYP2D6. This purified CYP2D6 catalyzed the N-demethylation of IMI and the 2-hydroxylation of DMI, but its ability to 2- or 10-hydroxylate IMI was not determined. There is one report in the literature on the metabolism of IMI in a homogenate of COS-1 cells in which human CYP2D6 was expressed (Brøsen *et al.* 1991). Metabolites isolated in low yield were DMI and 2-OH-IMI, together with trace amounts of 10-OH-IMI. In both studies, an HPLC analytical method was used to quantify the metabolites.

Although the cytochrome P450 isozymes that catalyze most of the N-demethylation of IMI to DMI have not been identified, some studies indicate that CYP2D6 contributes to a small extent to the N-demethylation of IMI *in vivo* (Brøsen & Gram 1989) and *in vitro* (Birgersson *et al.* 1986; von Bahr *et al.* 1989; Brøsen *et al.* 1991). An early study on the metabolism of IMI in man revealed that the rate of N-demethylation of IMI was faster in females than in males (Crammer *et al.* 1968). In contrast to this, faster N-demethylation was observed in the male rat than in the female (Pscheidt 1962). Thus, the isozyme catalyzing the N-demethylation of IMI exhibits sex differences in human and rat, and the enzymatic activity in this regard is reversed in the two species. More recently it was reported that CYP3A and CYP2C isozymes are male-specific forms in the rat (Imaoka *et al.* 1991; Smith 1991). Another study on human liver microsomes conducted by Hunt *et al.* (1992) found that the CYP3A activity was 24% higher in females than in males. It seems that CYP3A isozyme may be the enzyme responsible for N-demethylation of IMI and, if so, it would result in rate differences between females and males.

However, human *in vivo* studies indicated that age (Abernethy *et al.* 1985) and enzyme induction in alcoholics (Ciraulo *et al.* 1988) affect N-demethylation of IMI to a much greater extent than DMI hydroxylation. This suggested that CYP3A may not be the isozyme that catalyzes demethylation of IMI since enzymatic activity of the CYP3A is not affected by age, smoking status and ethanol consumption (Hunt *et al.* 1992).

Metabolism of several drugs such as antipyrine, oxazepam, and diazepam has been reported to exhibit a gender-related dimorphism in human and an age-related decline in clearance in males (Greenblatt *et al.* 1980 & 1982; Wilson 1984; Ochs *et al.* 1982; O' Malley *et al.* 1971). A human *in vivo* study suggested that N-demethylation of diazepam was catalyzed by a CYP2C isozyme since its N-demethylation correlated with S-mephenytoin 4-hydroxylation (Bertilsson *et al.* 1989). The fact that diazepam metabolism is also sex– and age– related is consistent with a CYP2C isozyme being involved. Six members of the human CYP2C subfamily have been identified (2C8, 2C9, 2C10, 2C17, 2C18, 2C19) (Nebert *et al.* 1991). It has been shown that 2C9 and 2C18 are not involved in S-mephenytoin 4-hydroxylation (Cholerton *et al.* 1992). The CYP2C isozyme catalyzing S-mephenytoin 4-hydroxylation has not been identified. N-Demethylation of IMI was not inhibited by mephenytoin or diazepam (Skjecob & Brøsen 1992), suggesting that the CYP2C isozyme responsible for S-mephenytoin 4-hydroxylation is not involved in the N-demethylation of IMI.

In light of the above details and information included in Table 1.3 in Chapter 1 (Metabolic N-dealkylation) which suggested that members of the CYP3A, 2C and 2B subfamilies are the isozymes involved most frequently in N-demethylation of drugs, it is possible that the CYP2C subfamily is the one that catalyzes N-demethylation of IMI.

### 2.1.3 Analytical methods

Accurate information on identifying and quantifying the metabolites of IMI, especially its hydroxylated metabolites, required a highly sensitive and selective analytical method. Quantitative determinations of IMI and its metabolites have been made in plasma (Nage & Johansson 1975; Gram *et al.*

1977; DeVane & Jusko 1981), urine and feces (Crammer *et al.* 1968; Gram & Christiansen 1975), and human breast milk (Sovner & Orsulak 1979). The analytical methods employed in these studies included thin-layer chromatography (TLC), autoradiography and HPLC. Recently, an HPLC method capable of quantifying IMI and its metabolites in human liver microsomes was developed and reported by Zeugin *et al.* (1990). It had a minimum detection level of 500 pmol/ml. However, the instability of 2-hydroxymetabolites encountered in this method limited its use for quantification of 2-OH-DMI and 2-OH-IMI. Previous investigators (Weder & Bickel 1968) have employed acetylation in the isolation of IMI and its metabolites from biological samples, but the method they employed was a tedious one in which phenolic bases were separated from non-phenolic bases, and solutions of the former were dried before being acetylated with acetic anhydride in dry pyridine for a 3 hour period. To overcome these disadvantages, a combined gas chromatography/mass spectrometry (GC/MS) procedure has been developed for analysis of IMI and its metabolites formed in a purified human CYP2D6 enzyme preparation.

## 2.2 EXPERIMENTAL

### 2.2.1 Chemicals

DMI hydrochloride, 2-OH-IMI hydrochloride, 2-OH-DMI oxalate, and 10-OH-IMI base were gifts from Ciba-Geigy (Mississauga, Canada). Iprindole (IPR) hydrochloride (internal standard) was kindly provided by Wyeth (Taplow, Maidenhead, UK). Other chemicals purchased from various souces include IMI hydrochloride (Aldrich, Milwaukee, WI, USA); NADP sodium salt from yeast (Terochem, Edmonton, Canada); D-glucose-6-phosphate, monosodium salt and glucose-6-phosphate dehydrogenase Type XII from Torula yeast (Sigma, St.

Louis, MO, USA); acetic anhydride, analytical grade (Fisher Scientific, Ottawa, Canada); potassium hydrogen carbonate, analytical reagent (BDH, Toronto, Canada). Dichloromethane, diethyl ether and toluene were obtained from BDH (Toronto, Canada).

Human CYP2D6 microsomal protein and control microsomal protein were purchased from Gentest Corporation (Woburn, MA, USA): lot numbers 15, 18 and 20 for CYP2D6; lot numbers 8, 11 and 16 for control microsomes. The CYP2D6 microsomal protein was prepared from a human AHH-1TK +/– cell line, *i.e.* a human B-lymphoblastoid cell line transfected with specific human cDNA that encoded CYP2D6. The control microsomes are prepared from the same AHH-1 TK +/– cell line that had not been transfected with specific cDNA, but contained a low level of CYP1A1 activity. Upon arrival packed in dry ice, the microsomal products were thawed rapidly in a 37°C waterbath with gentle agitation and placed on ice. The thawed products were divided into aliquots in a cold room (4°C) and stored at -80°C until used in metabolic studies. Human CYP1A1 (lot number 1) and CYP3A4 (lot number 6) were also purchased from Gentest Corporation and treated in the same manner as the CYP2D6 isozyme.

### 2.2.2 Assays

In brief, the method consisted of incubation for a suitable length of time, then direct acetylation of the incubation mixture using acetic anhydride under slightly basic conditions followed by extraction with diethyl ether/dichloromethane (14/11, v/v). After evaporation of the solvent, the residue was dissolved in 50 μl of toluene and an aliquot (1μl) was injected into the GC. IMI and its three metabolites produced by purified human cytochrome CYP2D6 isozyme were then identified with GC/MS and quantified with a GC method.

### 2.2.2.1 Incubation

Metabolism studies were conducted in KIMAX glass culture tubes (16 mm o.d. x 100 mm). The incubation was conducted in 0.1 M potassium phosphate buffer, pH 7.4 in the presence of a NADPH-generating system. The final concentrations of each component of the NADPH-generating system in the incubation mixture were 1.3 mM $NADP^+$ (1 mg/ml), 3.3 mM glucose-6-phosphate (1 mg/ml), 1 U/ml glucose-6-phosphate dehydrogenase (20 µl/ml) and 3.3 mM magnesium chloride ($MgCl_2.6H_2O$, 0.67 mg/ml). A solution of each component was prepared separately on ice before adding aliquots to the incubation mixture: $NADP^+$ and glucose-6-phosphate were prepared in buffer (pH 7.4) while $MgCl_2.6H_2O$ was dissolved in water. Glucose-6-phosphate dehydrogenase solution was prepared according to the supplier's instruction and was used directly.

In a typical incubation, 40 nmol substrate and 0.5 mg CYP2D6 microsomal protein in potassium phosphate buffer (pH 7.4) were preincubated in a culture tube at 37°C for 5 min before the addition of the NADPH-generating system. The resulting incubation mixture (total volume of 1 ml) was incubated for 30 min at 37°C in a shaking waterbath in air.

### 2.2.2.2 Work-up without acetylation

The incubation was stopped by addition of 100 µl of 25% potassium carbonate on ice, followed by 3 ml of diethyl ether/dichloromethane (14/11, v/v). The tube contents were mixed for 5 min using a multitube vortex mixer (IKA-Vibrax-VXR, Terochem, Edmonton, Canada) at the speed produced with a motor setting of 1400. After centrifugation at 1000g for 5 min, 2 ml of the organic phase were separated. The incubation mixture was extracted in the same way two more times, and the combined organic phase (6 ml) was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 25 µl toluene. An aliquot (1 µl) of this solution was injected into the GC

### 2.2.2.3 Work-up involving acetylation

The aqueous acetylation procedure was a modification of the method described by Torok-Both (1987). The incubation was stopped by rapid cooling of the culture tube in ice, followed quickly by addition of 100 µl of internal standard (IPR) solution (10 ng/µl) and potassium bicarbonate (350 mg). To the basified metabolic solution, 300 µl of acetic anhydride was added at room temperature. After the effervescence had ceased, the reaction mixture was extracted three times with 3 ml of diethyl ether/dichloromethane (14/11, v/v) by shaking for 5 min and then centrifuged for 5 min. The separated combined organic phase (6 ml) was evaporated under a stream of nitrogen and the dried extract was reconstituted in 50 µl of toluene. One µl of this final solution was used for GC analysis.

### 2.2.2.4 Instrumental analysis

A HP 5730A gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a nitrogen-phosphorus detector (NPD) and a DB-17 fused silica capillary column (20 m x 0.32 mm I.D. x 0.5 mm film thickness) was used for the separation of analytes. The initial column temperature of 180°C was held for 2 min, then increased to 280°C at a rate of 4°C/min and maintained at 280°C for 20 min. Ultra-pure helium (Union Carbide, Edmonton, Canada) was used as carrier gas at a flow rate of 1 ml/min. The injector and detector temperatures were 260°C and 310°C, respectively. Chromatograms were recorded with an HP 3396A integrator and peak areas were measured.

For confirmation of structures of metabolites, mass spectra were recorded in the electron-impact mode (ionization voltage 50 eV) using a VG-7070E mass spectrometer (VG Instruments, Manchester, UK) coupled to a Varian Vista 6000 gas chromatograph (Varian Instruments, Sunnydale, CA, USA). The GC column and temperature programs used for separation of analytes were the same as those used in the GC/NPD experiments. Helium was the carrier gas at a flow rate of 3 ml/min.

## 2.2.3 Quantification

### 2.2.3.1 Preparation of calibration curves

Five solutions, each containing IMI (10.7, 21.4, 32.1, 42.8 and 53.5 nmol), 2-OH-IMI (13.5, 10.1, 6.7, 3.4 and 1.7 nmol, respectively), DMI (4.1, 1.9, 3.0 5.7 and 0.75 nmol, respectively) and 2-OH-DMI (0.36, 0.89, 1.8, 2.7 and 3.6 nmol, respectively), were prepared in potassium phosphate pH 7.4 buffer and 0.5 mg of CYP2D6 microsomal preparation was added to each of the solutions to make the final volume of 780 µl. The incubations were carried out at 37°C for 5 min. To prevent any metabolism, each solution was rapidly cooled in ice before the addition of the internal standard (IPR, 100 µl), potassium bicarbonate (350 mg) and the NADPH-generating system (220 µl). The acetylation of each incubation solution was carried out in the same way as described in section 2.2.2.3. The final reconstituted solutions in toluene were used for GC analysis. Ratios of IMI/IPR peak areas *vs* IMI concentration; ratios of N-acetyl-DMI/IPR peak areas *vs* DMI concentration; ratios of 2-acetoxy-IMI/IPR peak areas *vs* 2-OH-IMI concentration; and ratios of 2-acetoxy-N-acetyl-DMI/IPR peak areas *vs* 2-OH-DMI concentration were plotted.

#### 2.2.3.2 Quality control

A solution with known concentrations of IMI (35.71 nmol), DMI (0.94 nmol), 2-OH-IMI (4.22 nmol) and 2-OH-DMI (1.77 nmol), and containing CYP2D6 isozyme and NADPH-generating system was incubated and acetylated in the same way as described above for preparation of a calibration curve, and was analyzed in triplicate. The concentration of each component in the solution was then determined using calibration curves constructed as described above.

### 2.2.4 *In vitro* metabolism study

#### 2.2.4.1 Effect of enzyme concentration

The standard incubation described in section 2.2.2.1 was performed on IMI, except that different quantities of CYP2D6 isozyme were used (0.25, 0.5, 1.0 and 1.5 mg).

#### 2.2.4.2 Effect of substrate concentration

The standard incubation described above in section 2.2.2.1 was repeated on IMI, except that substrate amounts were varied. The exact amounts were as follows: 9.8, 14.3, 40.0, 78.2, 114.3, 156.4 nmol for measurement of 2-OH-IMI and DMI; and 7.1, 28.5, 40.0, 57.1, 114.1 and 156.4 nmol for measurement of 2-OH-DMI.

#### 2.2.4.3 Effect of duration of incubation

The standard incubation described above in section 2.2.2.1 was repeated on IMI, except that different incubation times (5, 10, 20, 30 and 60 min) were employed.

#### 2.2.4.4 Comparison of N-dealkylation and C-hydroxylation pathways

To determine the preferred metabolic pathway which involved CYP2D6, IMI (40 nmol/ml), DMI (40 nmol/ml) and 2-OH-IMI (40 nmol/ml) were each subjected to incubation with CYP2D6 and cofactors, following the standard incubation procedure described above in section 2.2.2.1.

#### 2.2.4.5 Evaluation of CYP3A4 and CYP1A1 activity on N-demethylation of IMI

To see if CYP3A4 or CYP1A1 was involved in N-demethylation of IMI to any extent, IMI (40 nmol/ml) was subjected to incubation with CYP3A4 or CYP1A1 following exactly the same procedure as described above in section 2.2.2.1.

## 2.3 RESULTS

### 2.3.1 Separation of imipramine and its metabolites

#### 2.3.1.1 Acetylation technique

The *in vitro* metabolism of IMI by purified human CYP2D6 isozyme fortified with appropriate cofactors, produced 2-OH-IMI as its major metabolite, DMI in significant quantities, and 2-OH-DMI in trace amounts. No metabolites were detected when IMI was incubated with control microsomes, which were produced from a AHH–1 TK+/– cell line containing no vector and without a cDNA insert and containing native enzymatic activities, under the identical conditions as for CYP2D6 (Figure 2.3c). Using IPR as internal standard, IMI and its three metabolites in acetylated forms are very well separated on a DB-17 fused

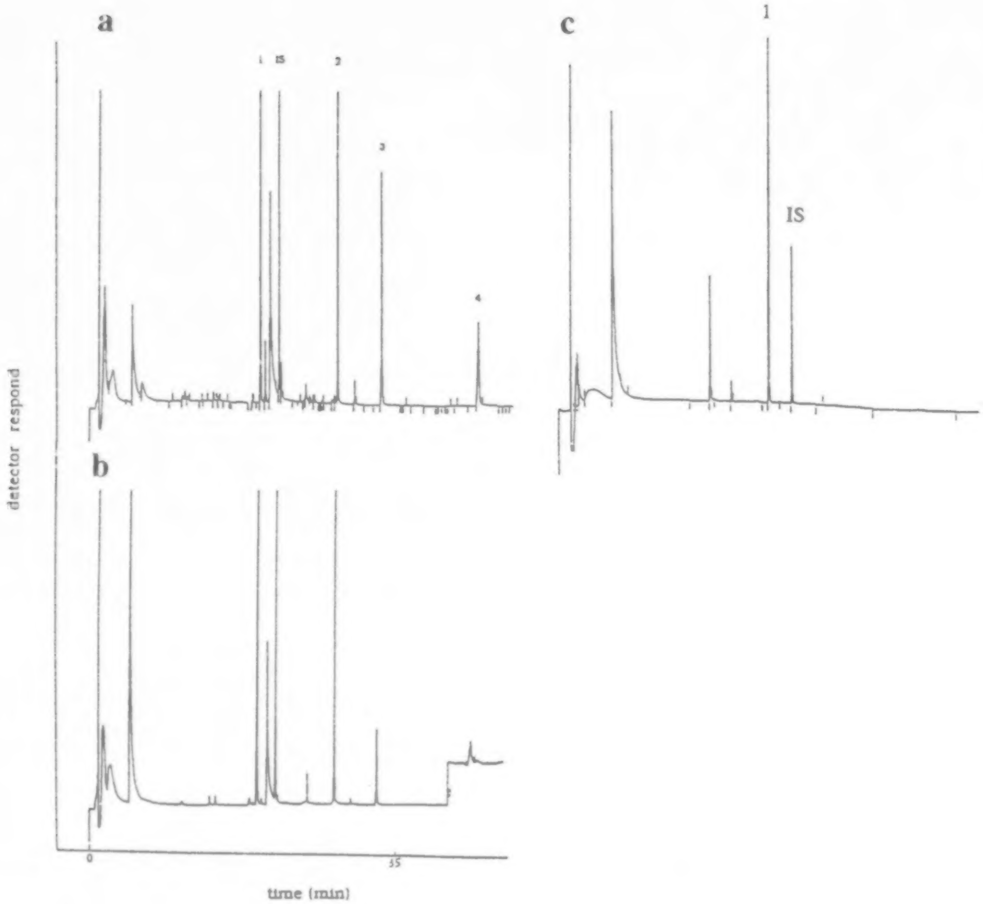


Figure 2.3 GC trace of: a) an acetylated extract of an incubation solution containing authentic samples of IMI (peak 1, $t_R$ = 19.13 min), IPR (IS, $t_R$ = 21.23 min), 2-acetoxy-IMI (peak 2, $t_R$ = 27.73 min), N-acetyl-DMI (peak 3, $t_R$ = 32.73 min), and 2-acetoxy-N-acetyl-DMI (peak 4, $t_R$ = 43.56 min); b) an acetylated extract of an IMI metabolism solution incubated with CYP2D6; and c) an acetylated extract of an IMI solution incubated with control microsomes.

silica capillary column and detected by an NPD (Figure 2.3b). Additional non-interfering peaks were routinely observed in the GC traces of extracts from both CYP2D6 and control incubations after the acetylation procedure. All of these may be attributable to other components in the metabolism solution that were not derived from IPR, IMI or its metabolites.

#### 2.3.1.2 Technique not utilizing acetylation

A GC trace of an extract of an IMI incubation mixture that had not undergone acetylation is illustrated in Figure 2.4b.

### 2.3.2 Identification of imipramine and its metabolites

#### 2.3.2.1 Acetylation technique

To identify the structures of all these three acetylated metabolites, *i.e.* 2-acetoxy-IMI, N-acetyl-DMI and 2-acetoxy-N-acetyl-DMI, their GC retention times (Figure 2.3b) and mass spectra (Figure 2.5) were compared with those of acetylated authentic samples of 2-OH-IMI, DMI and 2-OH-DMI, respectively (Figure 2.3a). Their mass spectra were also identical to published spectra (Maurer 1984). Appropriate structures for major fragmentation ions in each mass spectrum in Figure 2.5 are illustrated in Figure 2.6.

Another possible metabolite, 10-OH-IMI, detected as a trace metabolite in a homogenate of COS-1 cells transfected with cDNA that encoded human *CYP2D6* (Brøsen 1991), was not produced in the present study. The absence of 10-OH-IMI from the current incubation mixtures was confirmed by comparing the GC behavior of the IMI incubation mixture with that of an authentic sample of 10-OH-IMI, before and after acetylation using the conditions employed in the IMI metabolism experiments. When underivatized pure 10-OH-IMI was analyzed by

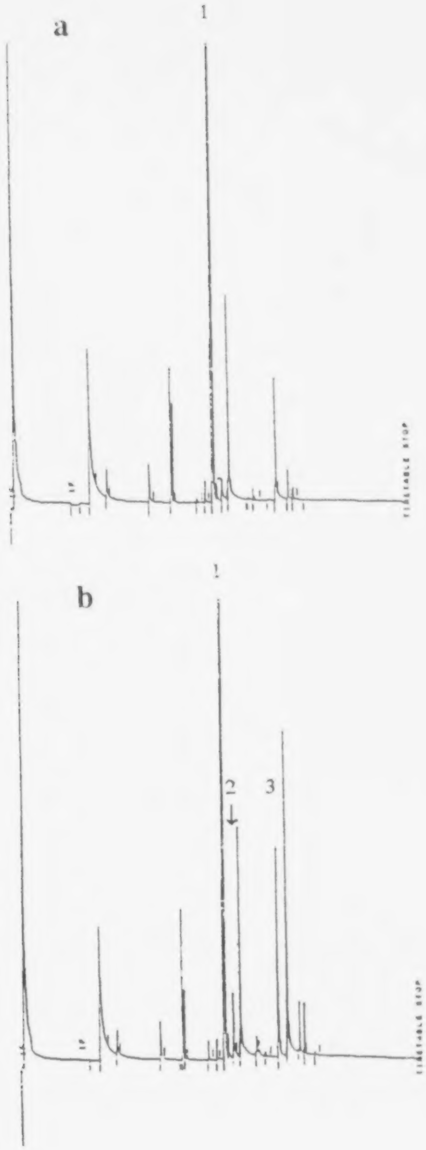
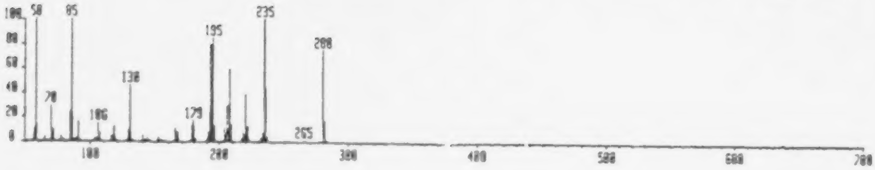
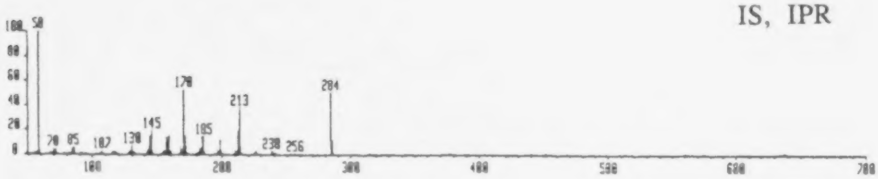


Figure 2.4 GC traces of a) an extract from a control microsomal incubation with IMI (80 μM) and b) an extract from an incubation of CYP2D6 with IMI (80 μM). Peak identification: (1) IMI, $t_R = 15.49$ min, (2) DMI, $t_R = 16.07$ min, (3) 2-OH-IMI, $t_R = 19.42$ min.

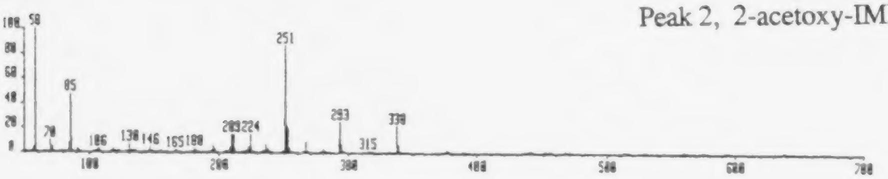
Peak 1, IMI



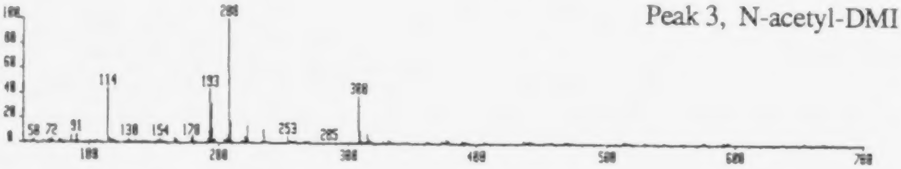
IS, IPR



Peak 2, 2-acetoxy-IMI



Peak 3, N-acetyl-DMI



Peak 4, 2-acetoxy-N-acetyl-DMI

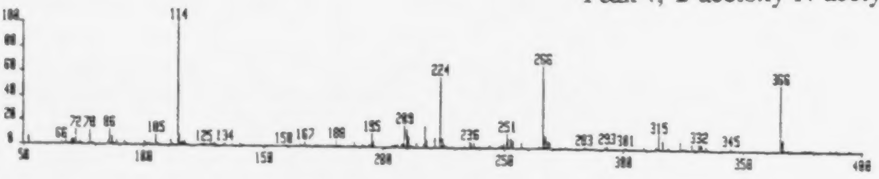
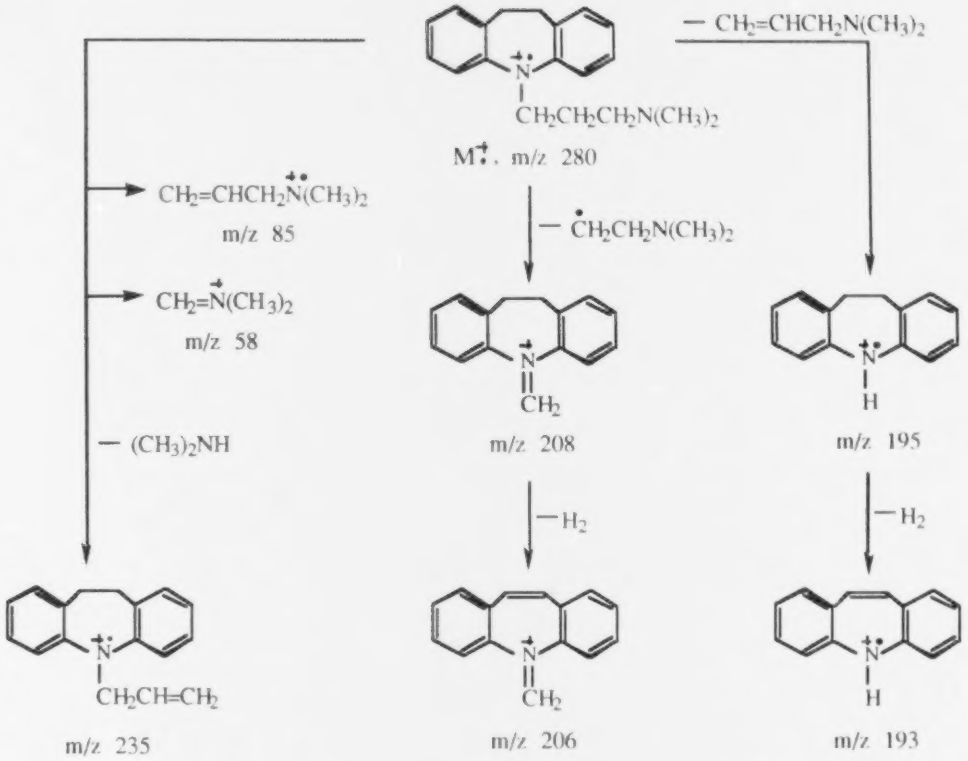
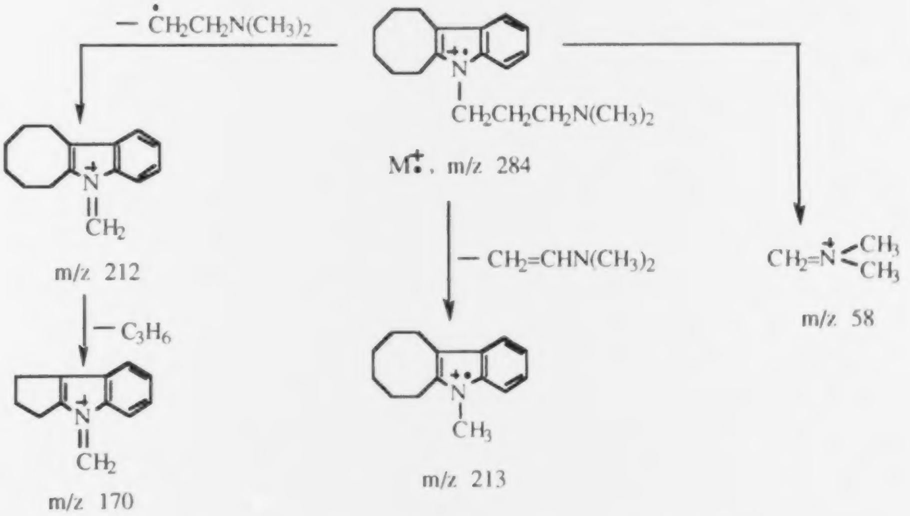


Figure 2.5 Mass spectra of IMI (peak 1 in Figure 2.3b) and its metabolites (peak 2, 3 and 4 in Figure 2.3b) obtained from an acetylated extract of a solution incubated with CYP2D6.

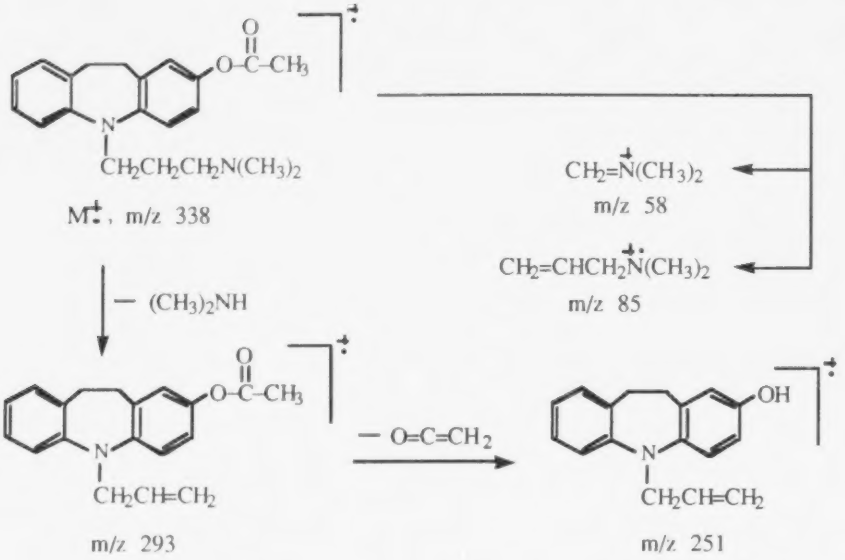

— CH2=CHCH2N(CH3)2
CH2CH2CH2N(CH3)2
M+•, m/z 280
CH2=CHCH2N(CH3)2
m/z 85
— •CH2CH2N(CH3)2
CH2=N(CH3)2
m/z 58
CH2
m/z 208
H
m/z 195
— (CH3)2NH
— H2
— H2
CH2CH=CH2
m/z 235
CH2
m/z 206
H
m/z 193

a) Mass fragmentation pathways of IMI.

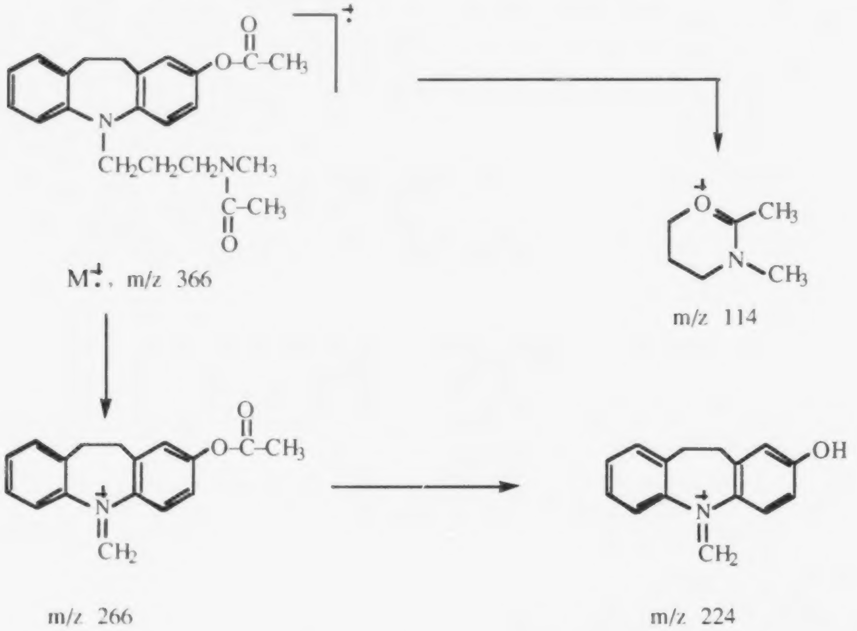

— •CH2CH2N(CH3)2
CH2CH2CH2N(CH3)2
M+•, m/z 284
CH2
m/z 212
— CH2=CHN(CH3)2
CH2=N<CH3 CH3
m/z 58
— C3H6
CH2
m/z 170
CH3
m/z 213

b) Mass fragmentation pathways of IPR.

**Figure 2.6** Mass fragmentation pathways of a) IMI, b) IPR, c) 2-acetoxy-IMI, d) 2-acetoxy-N-acetyl-DMI and e) N-acetyl-DMI.

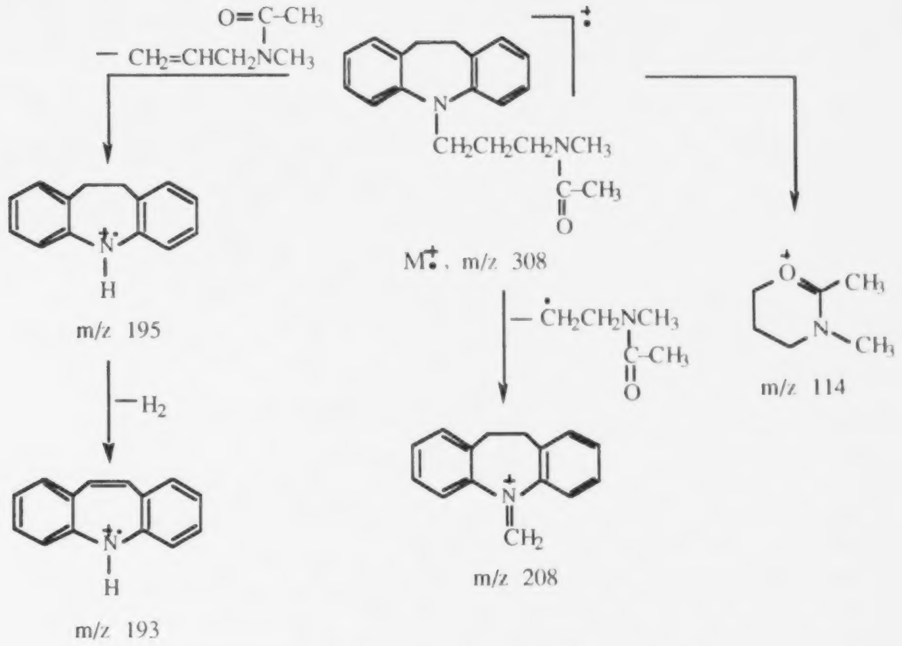

O-C-CH3
CH2CH2CH2N(CH3)2
M+•, m/z 338
CH2=N+(CH3)2
m/z 58
CH2=CHCH2N+•(CH3)2
m/z 85
— (CH3)2NH
O-C-CH3
CH2CH=CH2
m/z 293
— O=C=CH2
OH
CH2CH=CH2
m/z 251

c) Mass fragmentation pathways of acetylated 2-OH-IMI.


O-C-CH3
CH2CH2CH2NCH3
C-CH3
O
M+•, m/z 366
O+
CH3
N
CH3
m/z 114
O-C-CH3
N+
CH2
m/z 266
OH
N+
CH2
m/z 224

d) Mass fragmentation pathways of acetylated 2-OH-DMI.

**Figure 2.6** *(Continued)*


— $CH_2$=$CHCH_2NCH_3$
O=C-$CH_3$
$CH_2CH_2CH_2NCH_3$
C-$CH_3$
O
N
M$^{+\bullet}$, m/z 308
m/z 195
— $H_2$
m/z 193
— $\dot{C}H_2CH_2NCH_3$
m/z 208
$CH_2$
m/z 114
$CH_3$

e) Mass fragmentation pathways of acetylated DMI.

**Figure 2.6** *(Continued)*

GC/MS, two GC peaks were observed with $t_R$ values of 10.80 and 7.35 min. The mass spectra of these peaks (peak A and peak B, respectively) were recorded (Figure 2.7) and interpreted (Figure 2.8). The interpretation of the mass spectra revealed that peak A was 10-OH-IMI, while peak B was the dehydrogenated product, 10,11-dehydro-IMI. This indicated that some dehydration of 10-OH-IMI had occurred "on-column" when pure 10-OH-IMI was directly injected into the GC. When the sample of acetylated 10-OH-IMI was analyzed by GC/MS, five peaks were observed (Figure 2.9). Three of them were identified as 10-OH-IMI (peak A), 10-acetoxy-IMI (peak C; $t_R$ 11.67 min) and 10,11-dehydro-IMI (peak B) from their mass spectra (Figure 2.7). Fragmentation pathways compatible with these structures, and fragments common to all of them are provided in Figure 2.8.

Examination of the spectra of peaks D ($t_R$ 6.60 min) and E ($t_R$ 7.68 min) suggested that the analytes D and E were structurally related to 10-OH-IMI since the fragment ions of m/z 251, 233, 220, 210 and 192 in the spectrum of peak D were closely related in mass to ions of m/z 251, 233 and 232, 218, 207 and 206, and 193 in the spectrum of 10-OH-IMI (peak A) (Figure 2.7). Analyte E (m.w. 293) was deduced to be the acetylated derivative of analyte D (m.w. 251) based on their spectral similarity and the mass difference of their molecular ions. In addition, the absence of ions of m/z 58 and 85 revealed that the $CH_2CH_2CH_2N(CH_3)_2$ side-chain of analytes A, B and C had been modified in the formation of analytes D and E from A. Only an intact side chain attached to the ring N atom can give rise to ions of m/z 85 and 58 (Figure 2.8). The odd mass of the molecular ion of D is an indication of the presence of a single N atom (the ring N) in this degradation molecule. Conversion of A to analyte D required the expulsion of a fragment of 45 amu that contains an N atom; this can only be $(CH_3)_2NH$. The possible structures of analyte D could be IIIa or IVa as illustrated in Figure 2.10. If the structure of analyte D was IIIa, the initial fragmentation in its mass spectrum would be the expulsion of $H_2O$ to produce an ion of m/z 233, identical to the ion of that mass in the spectra of A, B and C. Following exactly the same procedure as depicted in Figure 2.8, this ion should fragment further to ions of m/z 218, 207 and 206; however, these ions were essentially not observed in the spectrum of analyte D. Also, it is difficult to explain the abundance of the ion of m/z 192 as base peak if analyte D possessed structure IIIa since this ion was only presented in low abundance in the spectra of A, B and C (Figure 2.7). Therefore, the structure of analyte D is more likely to be IVa and not IIIa, and the structure of analyte E will be IVb. The analytes D and E, therefore, are deduced to be the N-(1-propenyl) analog of 10-OH-IMI and its O-acetyl derivative as depicted as in Figure 2.11. It is apparent from these observations that alicyclic compounds such as 10-OH-IMI undergo chemical degradation during the relatively mild processes of aqueous acetylation and subsequent gas chromatography. Peaks with GC retention times corresponding to these five components (A to E) were absent from the GC trace of the extract obtained in the incubation of IMI.

### 2.3.2.2 Technique not utilizing acetylation

Structures of IMI and its metabolites, *i.e.* 2-OH-IMI, DMI and 2-OH-DMI, directly extracted from incubation mixtures without acetylation were confirmed by comparing their GC retention times (Figure 2.4b) with those of authentic samples of IMI, 2-OH-IMI, DMI and 2-OH-DMI, by interpretation of their mass spectra (Figure 2.12), and by comparing them with published spectra (Ardrey *et al.* 1985). Proposed structures for major fragmentation ions in each mass spectrum in Figure 2.12 are shown in Figure 2.13.

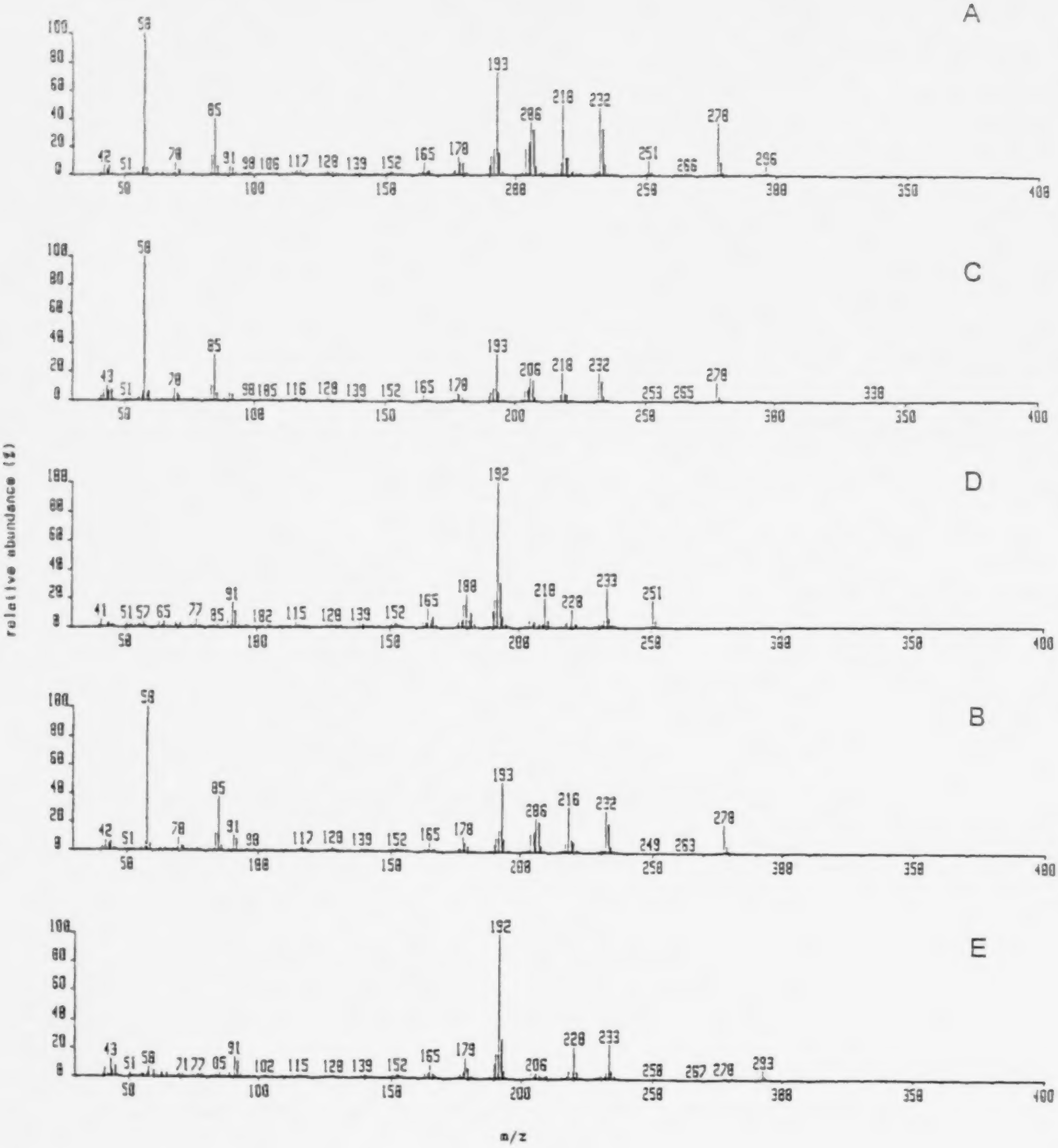


Figure 2.7 Mass spectra of 10-OH-IMI (peak A in Figure 2.9) and its degradation products (peaks B to E in Figure 2.9).

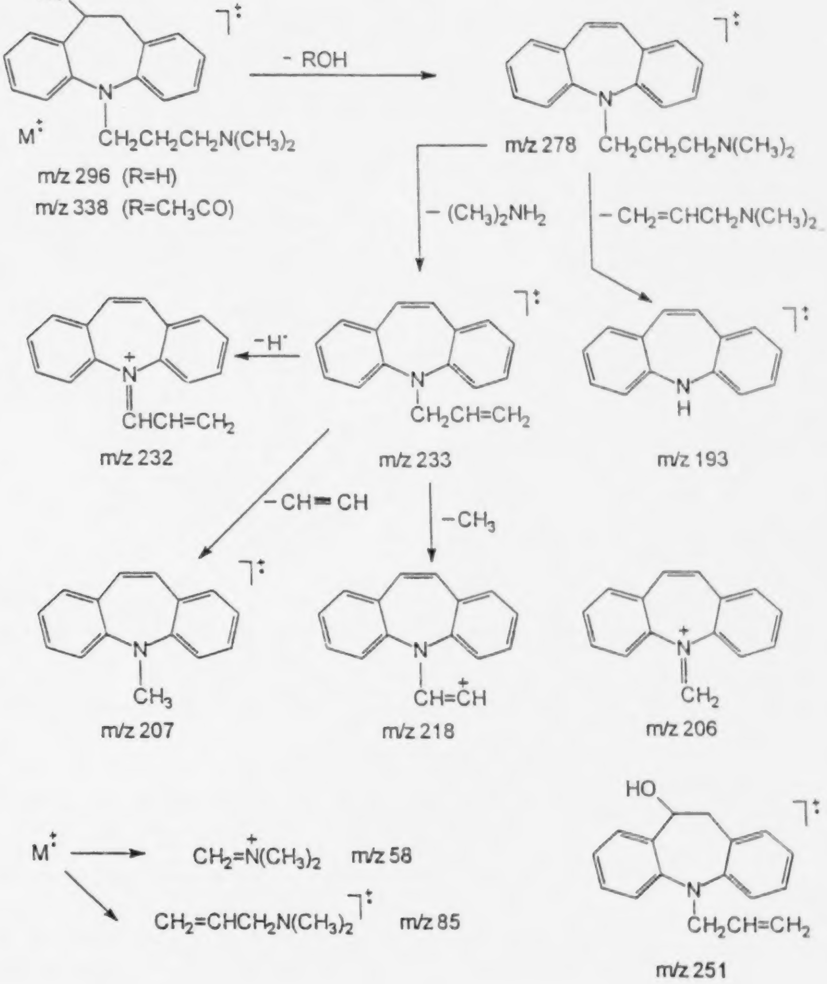
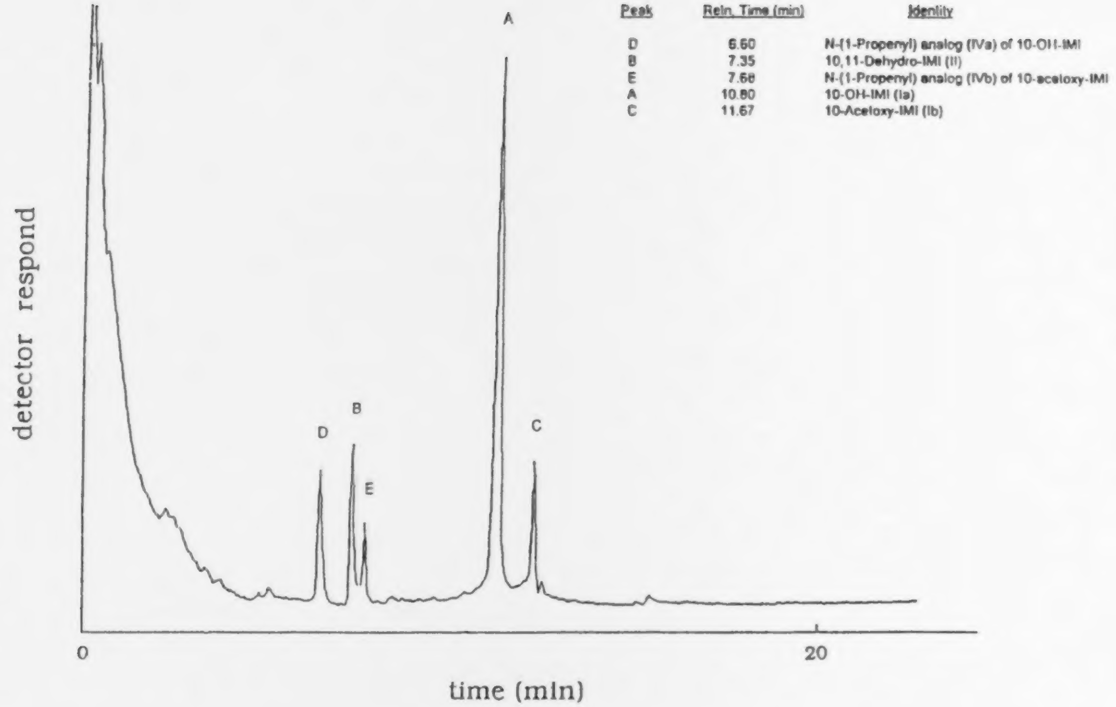
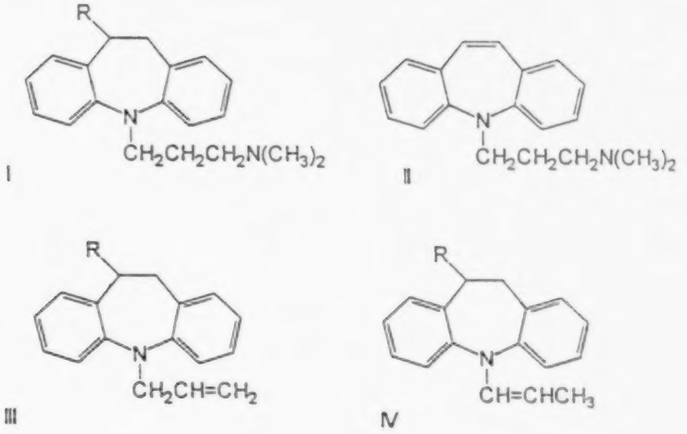

RO
M⁺˙
CH₂CH₂CH₂N(CH₃)₂
m/z 296 (R=H)
m/z 338 (R=CH₃CO)
- ROH
m/z 278
CH₂CH₂CH₂N(CH₃)₂
– (CH₃)₂NH₂
– CH₂=CHCH₂N(CH₃)₂
-H˙
CHCH=CH₂
m/z 232
CH₂CH=CH₂
m/z 233
H
m/z 193
–CH═CH
–CH₃
CH₃
m/z 207
CH=CH
m/z 218
CH₂
m/z 206
HO
M⁺˙
CH₂=N(CH₃)₂ m/z 58
CH₂=CHCH₂N(CH₃)₂ m/z 85
CH₂CH=CH₂
m/z 251

Figure 2.8 Common fragmentation ions in the mass spectra of 10-OH-IMI (m.w. 296, peak A in Figure 2.9); 10-acetoxy-IMI (m.w. 338, peak C in Figure 2.9) and 10,11-dehydro-IMI (m.w. 278, peak B in Figure 2.9). [The ion of m/z 206 may arise from either of ions m/z 278 or 233. The ion of m/z 251 is present only in the spectrum of D].



| Peak | Retn. Time (min) | Identity |
|---|---|---|
| D | 6.60 | N-(1-Propenyl) analog (IVa) of 10-OH-IMI |
| B | 7.35 | 10,11-Dehydro-IMI (II) |
| E | 7.68 | N-(1-Propenyl) analog (IVb) of 10-acetoxy-IMI |
| A | 10.80 | 10-OH-IMI (Ia) |
| C | 11.67 | 10-Acetoxy-IMI (Ib) |

**Figure 2.9** GC trace obtained during the GC/MS analysis of an authentic sample of 10-OH-IMI which had been subjected to acetylation.


R
N
CH₂CH₂CH₂N(CH₃)₂
I
N
CH₂CH₂CH₂N(CH₃)₂
II
R
N
CH₂CH=CH₂
III
R
N
CH=CHCH₃
IV

a) R=OH; b) R=OCOCH$_3$

Figure 2.10 Structures of 10-OH-IMI and its degradation products.

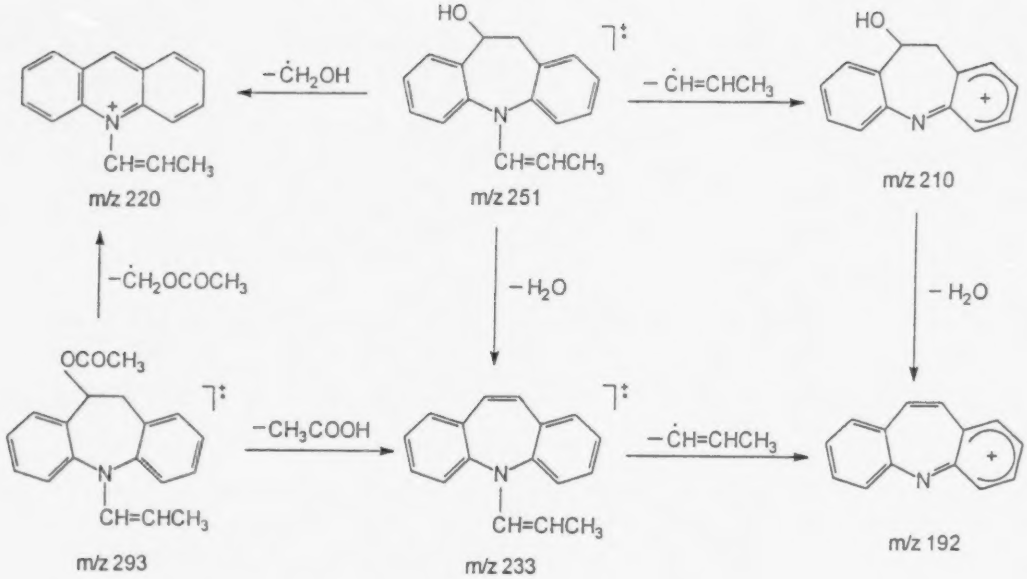

HO
m/z 220
m/z 251
m/z 210
-ĊH2OH
- ĊH=CHCH3
-ĊH2OCOCH3
- H2O
- H2O
OCOCH3
-CH3COOH
- ĊH=CHCH3
CH=CHCH3
m/z 293
m/z 233
m/z 192

Figure 2.11 Proposed mass spectral fragmentation pathways of an N-(1-propenyl) analog of 10-OH-IMI (m.w. 251, peak D in Figure 2.9) and its O-acetyl derivative (m.w. 293, peak E in Figure 2.9) formed as decomposition products during GC/MS analysis of acetylated A (in Figure 2.9).

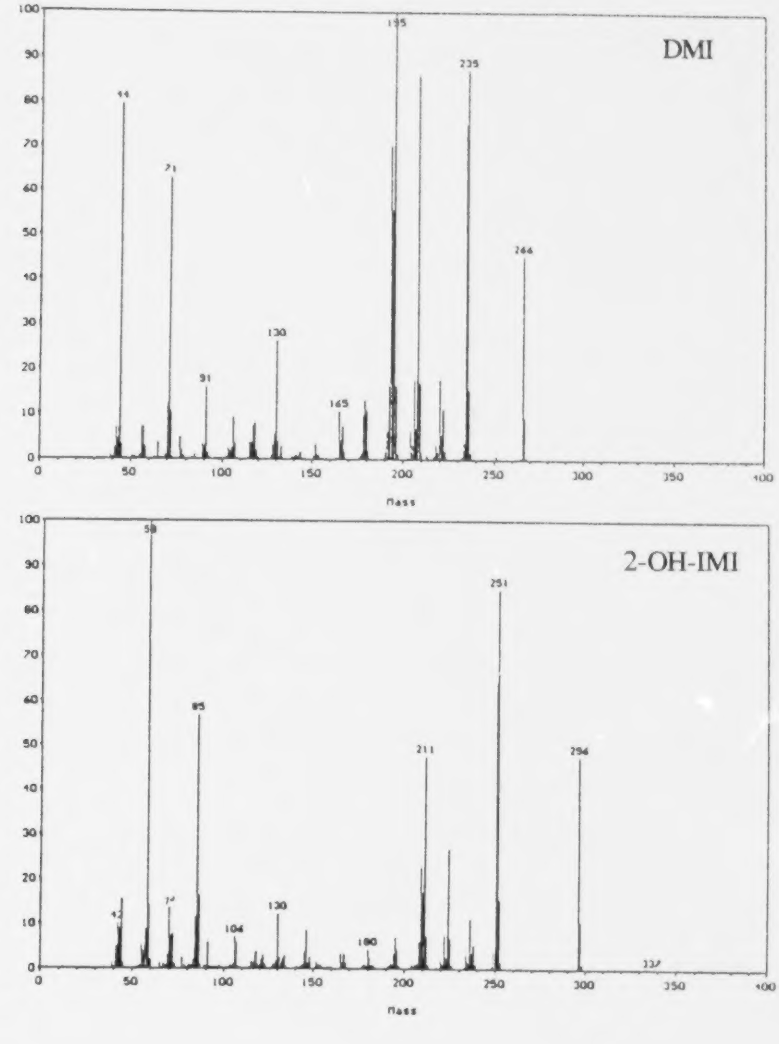
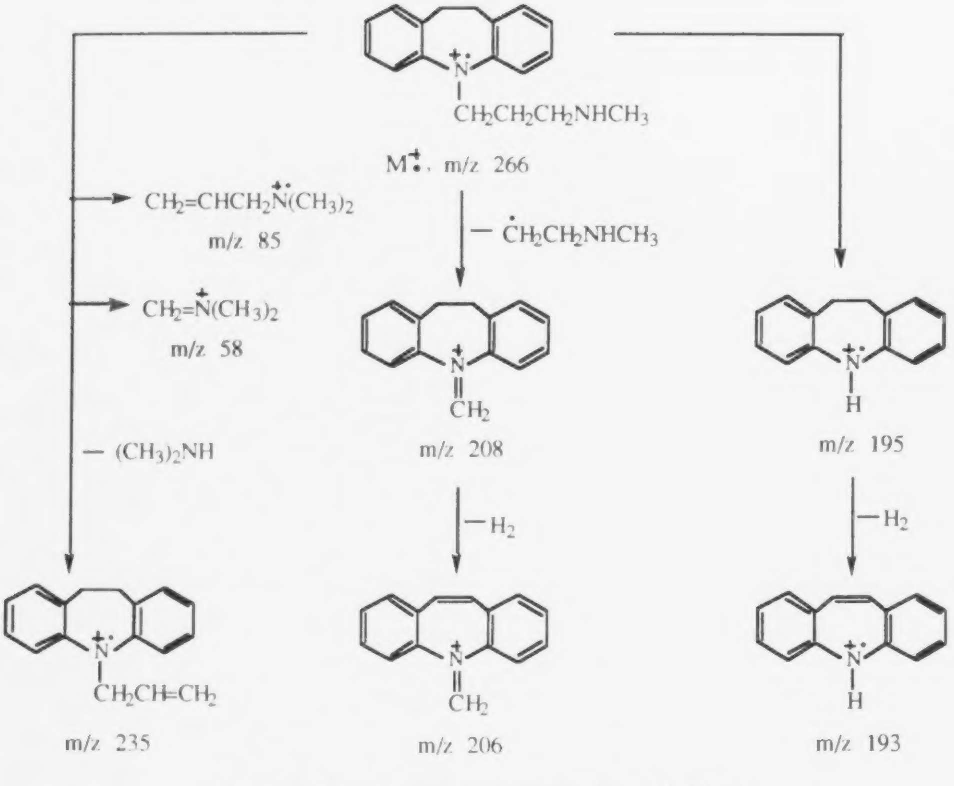
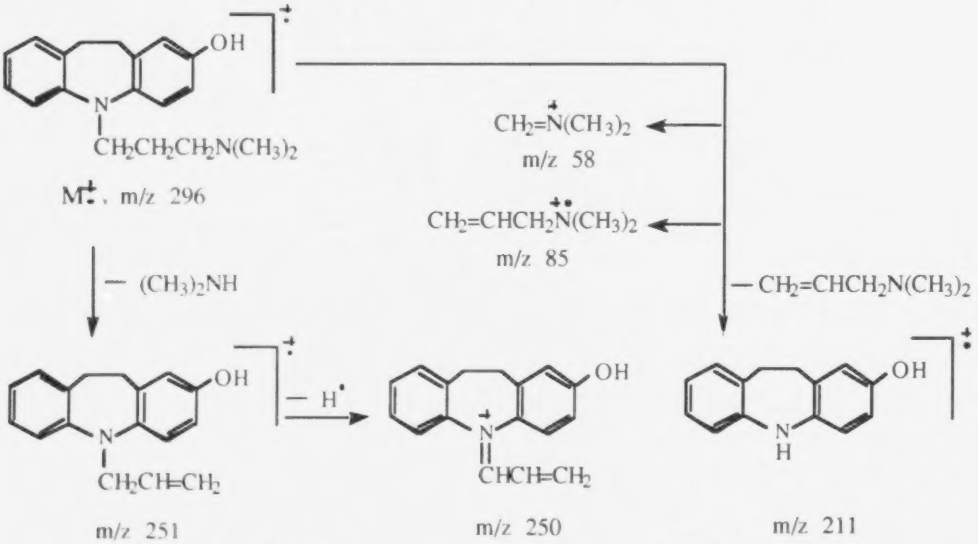


Figure 2.12 Mass spectra of DMI (peak 2 in Figure 2.4b) and 2-OH-IMI (peak 3 in Figure 2.4b) formed as metabolites of IMI from an CYP2D6 incubation mixture.


$CH_2CH_2CH_2NHCH_3$
M⁺˙, m/z 266
$CH_2{=}CHCH_2\overset{+\bullet}{N}(CH_3)_2$
m/z 85
$- \dot{C}H_2CH_2NHCH_3$
$CH_2{=}\overset{+}{N}(CH_3)_2$
m/z 58
$CH_2$
m/z 208
H
m/z 195
$- (CH_3)_2NH$
$-H_2$
$-H_2$
$CH_2CH{=}CH_2$
m/z 235
$CH_2$
m/z 206
H
m/z 193

a) Mass fragmentation pathways of DMI


OH
$CH_2CH_2CH_2N(CH_3)_2$
M⁺˙, m/z 296
$CH_2{=}\overset{+}{N}(CH_3)_2$
m/z 58
$CH_2{=}CHCH_2\overset{+\bullet}{N}(CH_3)_2$
m/z 85
$- (CH_3)_2NH$
$- CH_2{=}CHCH_2N(CH_3)_2$
OH
$- H^{\bullet}$
OH
OH
$CH_2CH{=}CH_2$
$CHCH{=}CH_2$
H
m/z 251
m/z 250
m/z 211

b) Mass fragmentation pathways of 2-OH-IMI.

**Figure 2.13 Mass fragmentation pathways of a) DMI and b) 2-OH-IMI.**

### 2.3.3 Assays

A gas chromatographic procedure for the quantification of IMI and its metabolites 2-OH-IMI, DMI and 2-OH-DMI was developed. The procedure required the acetylation of the incubation mixture to minimize the decomposition of 2-OH-IMI. Calibration graphs, *i.e.* IMI/IPR peak area ratios *vs* IMI concentration, N-acetyl-DMI/IPR peak area ratios *vs* DMI concentration, and 2-acetoxy-IMI/IPR peak area ratios *vs* 2-OH-IMI concentration, and 2-acetoxy-N-acetyl-DMI/IPR peak area ratio *vs* 2-OH-DMI concentration, were prepared over concentration ranges that simulated what had been observed in initial metabolism reactions conducted on IMI. The solutions prepared for the construction of these calibration graphs also contained CYP2D6 and the NADPH-generating system. Regression analysis demonstrated that these relationships were linear over the concentration ranges analyzed, with $r^2$ values always $> 0.99$. Calibration curves generated on different days were reproducible. The typical calibration curves gave the following equations: $y=0.571+0.232x$, $r^2=0.991$ for IMI; $y=0.0268+0.193x$, $r^2=0.998$ for DMI; $y=0.0378+0.111x$, $r^2=0.998$ for 2-OH-IMI and $y=0.00170+0.0281x$, $r^2=0.996$ for 2-OH-DMI. Reproducibility and accuracy of the analytical procedure were satisfactory (Table 2.1). The sensitivity of the method (the minimum quantifiable concentration) was evaluated as 1 µg/ml for IMI, 0.5 µg/ml for 2-OH-IMI, 0.2 µg/ml for DMI and 0.1 µg/ml for 2-OH-DMI. With a 4:1 signal:noise ratio, the lowest limit of detection on column would be 1 pmole for IMI and DMI, 3 pmoles for 2-OH-IMI and 50 pmoles for 2-OH-DMI. These results confirm that the developed analytical procedure was accurate, reproducible and sensitive. Extracts containing acetylated metabolites were stable for at least 10 weeks when stored at 4°C.

**Table 2.1 Accuracy and reproducibility of GC analysis of IMI and its metabolites in enzyme preparation (n=3)**

| Compound | Prepared concn. (nmol/ml) | Measured concn. (nmol/ml) | CV% | Error% |
|---|---|---|---|---|
| IMI | 35.71 | 34.81 | 5.1 | -2.5 |
| 2-OH-IMI | 4.22 | 4.15 | 7.3 | -1.7 |
| DMI | 0.94 | 0.99 | 10.7 | 5.3 |
| 2-OH-DMI | 1.77 | 1.73 | 10.4 | -2.3 |

### 2.3.4 Metabolism study

The effects of altering enzyme protein concentration, substrate concentration and duration of incubation on the yields of the three metabolites (2-OH-IMI, DMI and 2-OH-DMI) were determined in duplicate (Figure 2.14-2.16). Yields and rates of formation of these metabolites of IMI were then determined (Table 2.2).

#### 2.3.4.1 Effect of enzyme concentration on the yields of metabolites

Incubation of the same amount of IMI with increasing concentrations of CYP2D6 isozyme preparation (0.25-1.5 mg/ml) resulted in the formation of 2-OH-IMI, DMI and 2-OH-DMI in quantities that increased linearly with increasing isozyme concentration. Higher yields of 2-OH-IMI than DMI and 2-OH-DMI were obtained (Figure 2.14).

#### 2.3.4.2 Effect of substrate concentration on the yields of metabolites

When the same amount of CYP2D6 was incubated with increasing amounts of IMI (7.13-156.4 nmol), both 2-hydroxylation and N-demethylation of IMI became saturated (Figure 2.15). Again, the yield of 2-OH-IMI was much greater (6-fold) than that of DMI at saturation. The apparent $K_m$ and $V_{max}$ values of two of IMI's three metabolic reactions (Table 2.3) were determined graphically by construction of Lineweaver-Burk plots. A Lineweaver-Burk plot yielded a straight line, $1/V = 0.178 + 1.52 \times 1/[S]$ for 2-OH-IMI, and $1/V = 0.031 + 0.18 \times 1/[S]$ for DMI. $V_{max}$ values were calculated from each of these equations by

### Table 2.2 Metabolic hydroxylation and N-dealkylation of IMI*

| Metabolite | Yield (nmol) (mean ± SD) | % of initial substrate concn. (mean ± SD) | Rate of formation (nmol/mg/h) (mean ± SD) |
|---|---|---|---|
| 2-OH-IMI | 8.26 ± 0.77 | 21.55 ± 1.94 | 34.48 ± 3.10 |
| DMI | 1.06 ± 0.063 | 2.65 ± 0.16 | 4.24 ± 0.25 |
| 2-OH-DMI | 0.077 ± 0.009 | 0.20 ± 0.02 | 0.31 ± 0.042 |
| IMI (recovered) | 26.02 ± 2.19 | 65.06 ± 5.47 | |

*Standard incubation mixture: substrate = 40 nmol IMI/ml; CYP2D6 = 0.5 mg/ml; incubation time = 30 min; n = 3.

### Table 2.3 Apparent $K_m$ and $V_{max}$ values of IMI metabolism by CYP2D6

| Metabolic reaction | Apparent $K_m$ (µmol/L) | $V_{max}$ (nmol/mg/h) |
|---|---|---|
| IMI → DMI | 8.52 | 5.61 |
| IMI → 2–OH–IMI | 5.72 | 32.14 |

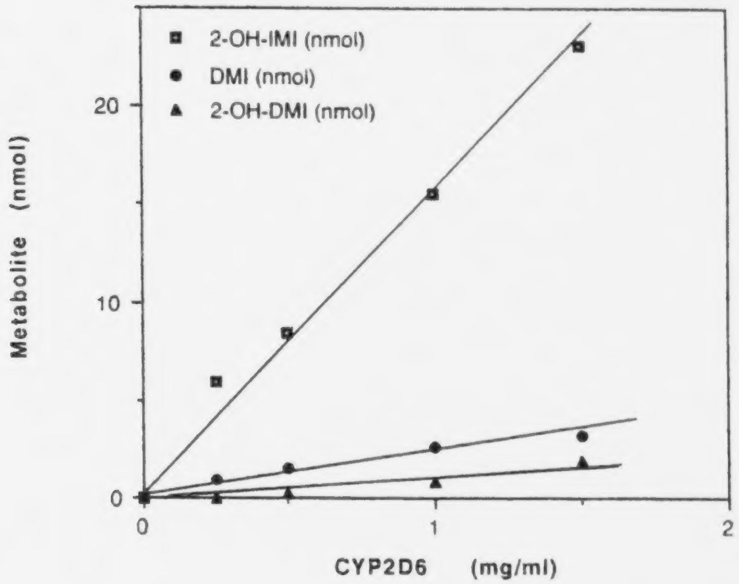


Figure 2.14 Dependence of the formation of IMI metabolites on CYP2D6 concentration.

[The incubation mixture (1 ml) contained substrate (40 nmol), CYP2D6 (range 0.25–1.5 mg) and cofactors (see section 2.2.2.1). Incubation was performed at 37°C for 30 min. Each point is the mean value of two experiments that differed by less than 5%].

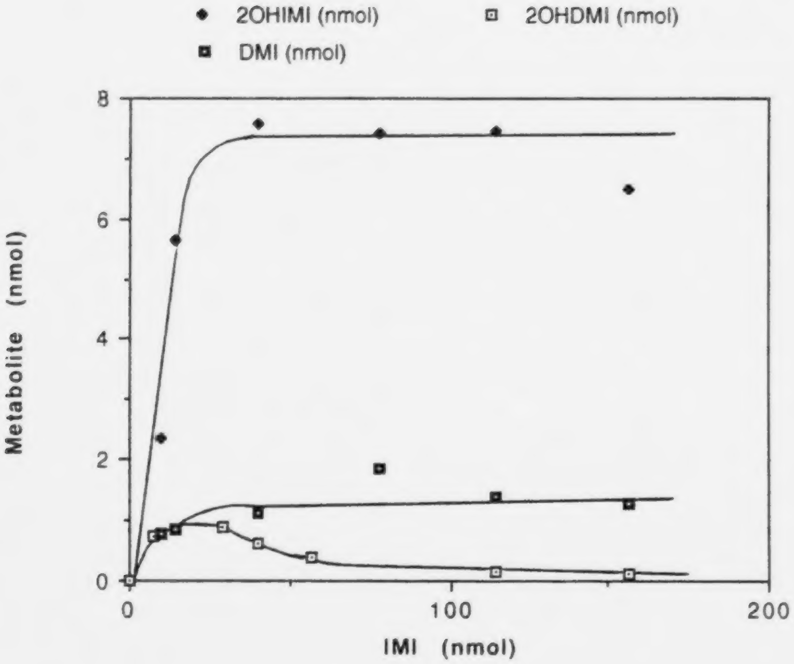


Figure 2.15 Effect of substrate concentration on the yields of IMI's metabolites.

[The incubation mixture (1 ml) contained substrate (range 7.1–156.4 nmol), CYP2D6 (0.5 mg) and cofactors (see section 2.2.2.1). Incubation was performed at 37°C for 30 min. Each point is the mean value of two experiments that differed by less than 5%.]

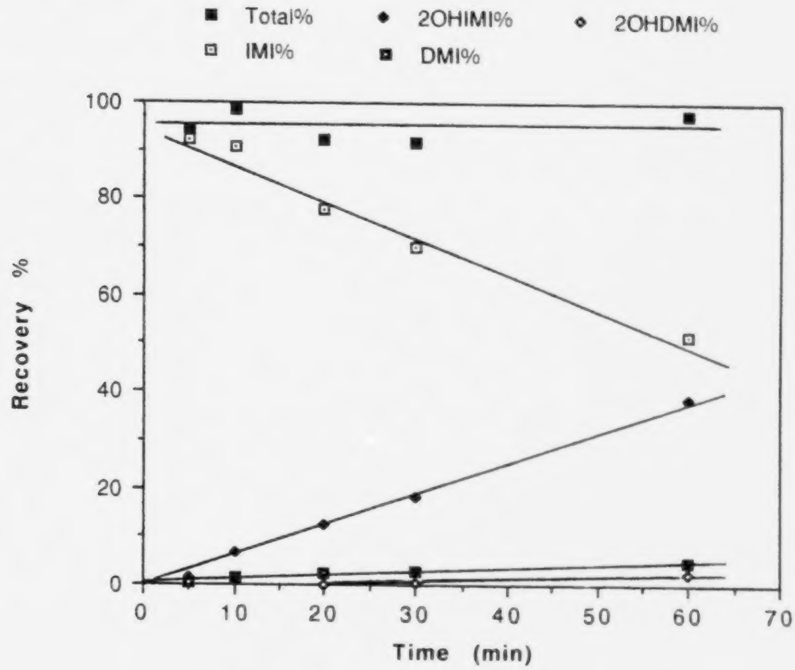


Figure 2.16 Relationship between recovered IMI and its metabolites.

[The incubation mixture (1 ml) contained substrate (40 nmol), CYP2D6 (0.5 mg) and cofactors (see section 2.2.2.1). Incubation was performed at 37°C for 5–60 min. Each point is the mean value of two experiments that differed by less than 5%.]

giving 1/[S] a value of zero. $K_m$ was then calculated from the equation, $K_m$ = slope x $V_{max}$. The apparent $K_m$ and $V_{max}$ values of 2-OH-DMI formation could not be determined since the formation of this metabolite did not follow saturation kinetics.

#### 2.3.4.3 Effect of duration of incubation on the yields of metabolites

Duration of incubation was varied from 5 to 60 min while keeping both the substrate concentration and CYP2D6 amount constant. The production of 2-OH-IMI and DMI increased with increasing incubation time. The recoveries of IMI, 2-OH-IMI, DMI and 2-OH-DMI are provided in Figure 2.16. 2-OH-IMI was the most abundant metabolite recovered at all time intervals examined, while 2-OH-DMI was the metabolite obtained in the lowest yield. This latter metabolite was not detectable until incubation had proceeded for 20 min. Total recoveries of unmetabolized IMI and its three metabolites were determined in each experiment and averaged 95.02%.

#### 2.3.4.4 Comparison of N-dealkylation and C-hydroxylation pathways

The formation of 2-OH-DMI from IMI can proceed *via* either DMI or 2-OH-IMI. To provide information on the preferred pathway, both 2-OH-IMI and DMI were subjected to metabolism by the human CYP2D6 isozyme preparation. The recoveries of 2-OH-DMI from both metabolic reactions are given in Figure 2.17. The yield of 2-OH-DMI recovered *via* aromatic hydroxylation of DMI was 4-fold higher than *via* initial N-dealkylation of 2-OH-IMI, indicating that aromatic hydroxylation was more efficient than N-dealkylation with the CYP2D6 system used.

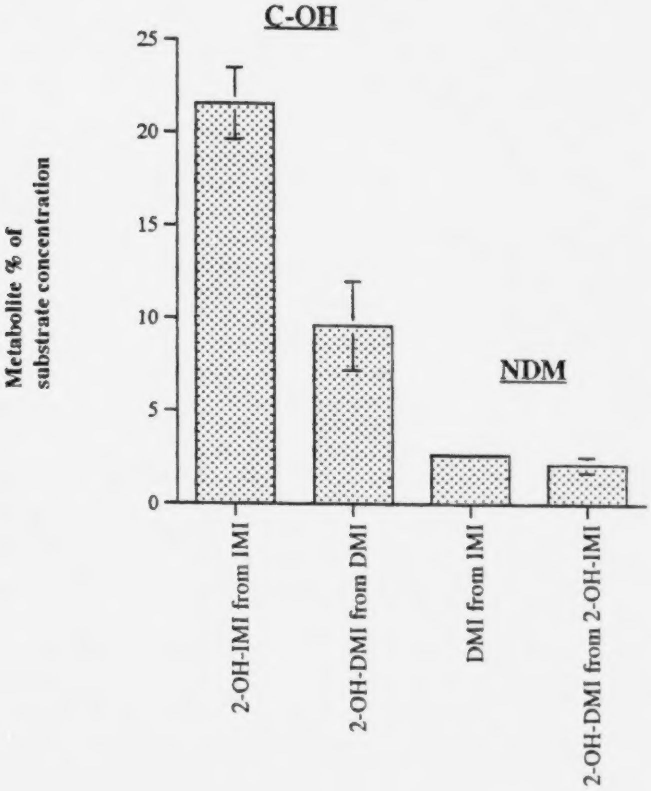


**Figure 2.17** Relative efficiencies of metabolic C-hydroxylation (C-OH) and N-demethylation (NDM).

#### 2.3.4.5 Evaluation of CYP3A4 and CYP1A1 activity on N-demethylation of IMI

No measurable DMI was formed with this microsomal preparation under the incubation conditions described in section 2.2.2.1.

## 2.4 DISCUSSION

A preliminary experiment on the analysis of IMI and its metabolites was conducted directly on the extracts of incubation mixtures that had not been acetylated. However, instability of 2-hydroxy metabolites was observed and this resulted in poor recovery of these metabolites and difficulties in quantifying them. Since these preliminary results were useful in the identification of these metabolites, this analytical procedure is also provided in section 2.2.2.2.

In the course of analysis of IMI and its metabolites, the instability of 2-OH-IMI was encountered by previous investigators (Zeugin *et al.* 1990) and also in our laboratory. When extracts containing IMI, 2-OH-IMI, DMI and internal standard (IPR) were stored for two weeks at 4°C then rechromatographed, the peak due to 2-OH-IMI was absent from the GC trace whereas the peaks due to IMI, DMI and IPR were unaffected. During the interval of two weeks, the 2-OH-IMI had completely decomposed. Attempts were made to prevent the decomposition of 2-OH-IMI. Addition of ascorbic acid, an antioxidant, to the solution prepared for GC analysis did not prevent the decomposition of 2-OH-IMI during storage. Purging the same solution with nitrogen prior to its storage was also unsuccessful in preserving 2-OH-IMI. When ethyl acetate was employed as the solvent for GC analysis of IMI and its metabolites, it was observed that the DMI component underwent "on column" acetylation to N-acetyl DMI. This was confirmed by examining the GC behavior of an ethyl acetate solution of pure DMI base. These analytical problems were overcome by immediately acetylating the incubation mixture with acetic anhydride at room temperature at the end of the incubation period. The acetylation was conducted in the aqueous medium of the incubation mixture in the presence of an excess of potassium bicarbonate. Previous investigators (Weder & Bickel 1968) have employed acetylation in the isolation of imipramine and its metabolites from biological samples, but the method they employed was a tedious one in which phenolic bases were separated from non-phenolic bases then dried before being acetylated with acetic anhydride in dry pyridine for a 3 h period.

Another analytical problem was encountered when initial attempts were made to construct calibration curves for the quantitation of 2-OH-IMI. When solutions containing known concentrations of IMI, DMI, 2-OH-IMI, cofactors and IPR but no enzyme were analyzed, a linear relationship for the plot of 2-O-acetoxy-IMI/IPR ratio *vs* 2-OH-IMI concentration was obtained, but when extracts of incubation mixtures were evaluated using this calibration curve, the measured concentrations of 2-OH-IMI were always unacceptably high at low concentrations of this metabolite. This problem was overcome by the inclusion of CYP2D6 enzyme in all the solutions used in calibration curve construction. All calibration graphs were then linear over the concentration ranges examined.

An unexpected observation was made in the process of confirmation of 10-OH-IMI as a metabolite of IMI in the purified CYP2D6 isozyme preparation. When subjected to acetylation, a pure sample of 10-OH-IMI partially underwent a combination of O-acetylation, 10,11-dehydrogenation and side-chain modification to produce four degradation products, thus complicating the analytical technique.

Fortunately 10-OH-IMI was not detected as a metabolite of IMI in this study, but in other investigations that require quantitation of 10-OH-IMI, the acetylation technique could not be used.

This study has shown that human CYP2D6 expressed in a human AHH-1 TK+/– cell line efficiently catalyzes the aromatic hydroxylation of IMI to 2-OH-IMI and also mediates IMI's N-dealkylation to DMI to a significant extent. A third metabolite, 2-OH-DMI, was also formed mainly from DMI, but only to a small extent. Previous investigations have shown that 2-OH-IMI and 2-OH-DMI are major *in vivo* metabolites of IMI in humans and that the latter metabolite is formed mainly from the primary metabolite, DMI. The metabolism of IMI *in vitro* produces different results. When Zeugin *et al.* (1990) and Brøsen *et al.* (1991) investigated the biotransformation of IMI with human liver microsomes, the products identified were 2-OH-IMI and DMI, and a small amount of 10-OH-IMI, but no 2-OH-DMI was detected. Metabolism of IMI by the microorganism *Mucor. griseo-cyanus* also produced DMI, 2-OH-IMI and 10-OH-IMI, but no 2-OH-DMI (Hufford *et al.* 1981).

When the present investigation of the *in vitro* metabolism of IMI with CYP2D6 expressed in a human AHH-1 TK+/– cell line was commenced, the anticipated result was that oxidation of IMI to 2-OH-IMI would be the major metabolic pathway observed since this biotransformation sequence is known to be catalyzed by CYP2D6 (Brøsen *et al.*1991; von Bahr *et al.* 1985; Steiner *et al.* 1987). It was also expected that small amounts of 10-OH-IMI would be formed in view of the investigations by others that have just been described. No N-dealkylation to DMI and 2-OH-DMI was expected because the N-dealkylation metabolic pathway is generally not associated with the CYP2D6 isozyme. However, in our study, biotransformation of IMI to 2-OH-IMI was observed but no 10-OH-IMI was detected. Somewhat surprisingly, N-demethylation of IMI to DMI occurred consistently, although to a lesser extent than 2-OH-IMI formation. That metabolic formation of DMI was indeed catalyzed by CYP2D6 can be inferred from the observation that DMI was detected in incubation mixtures containing the CYP2D6 microsomal protein, but was absent from incubation mixtures containing control microsomal protein which was produced from a cell line that had not been transfected with specific cDNA but contained native microsomal enzyme activity.

Incubations of a constant quantity of IMI with increasing concentrations of CYP2D6 isozyme resulted in linearly increasing production of all three metabolites (2-OH-IMI, DMI and 2-OH-DMI) (Figure 2.14), indicating that over the isozyme concentration range used, maximum yields of the three metabolites were obtained at each isozyme concentration, and that the isozyme preparation retained its viability. Therefore, any isozyme concentration within this range would be appropriate for a study to determine the effect of substrate concentration on the rate of formation of the three metabolites.

When a constant amount of CYP2D6 isozyme was incubated with increasing amounts of IMI, both 2-hydroxylation and N-demethylation reactions reached saturation (Figure 2.15) and the rate of formation of 2-OH-IMI was about six times faster than it was for DMI production to reach saturation (Table 2.3). When these results were compared with those obtained by Krüger *et al.* (1986) in their *in vitro* study on the metabolism of IMI with rat liver microsomes, an interesting difference was observed. These investigators found that hydroxylation of IMI to 2-OH-IMI was a saturable process whereas the rate of N-demethylation of IMI to DMI did not reach saturation over the same substrate concentration range. This suggested that formation of DMI from IMI by rat liver microsome involves another P450 isozyme in addition to CYP2D6.

When equal concentrations of IMI and DMI were incubated under identical conditions with the fortified CYP2D6 isozyme preparation, the yields of primary metabolites of IMI and DMI, *i.e.* 2-OH-IMI and 2-OH-DMI, were 21.6% and 9.6%, respectively (Figure 2.17). This indicated that IMI is the preferred substrate for CYP2D6. Such a conclusion is in agreement with similar observations by Krüger *et al.* (1986) using rat liver microsomes. Sutfin *et al.* (1984) also attribute the longer half-life of DMI over IMI in humans to the higher rate of hydroxylation of IMI, which is known to be catalyzed by CYP2D6.

IMI and 2-OH-IMI were subjected to metabolism with CYP2D6 to examine the efficiency of CYP2D6 to catalyze their N-demethylations (Figure 2.17). Both substrates were N-demethylated to a similar, though very small (2.6%) extent. When rat liver microsomes were used in a related study (Krüger *et al.* 1986), similar results were obtained. Both IMI and 2-OH-IMI underwent metabolic N-demethylation but the former reaction proceeded more efficiently. The contribution of CYP2D6 to these rat N-demethylation reactions was not determined.

In view of the fact that 2-OH-DMI is a major *in vivo* metabolite of IMI in humans (Potter & Calil 1981), further studies are required to identify other CYP isozymes that may be involved in the production of 2-OH-DMI. Apparently CYP2D6 is involved in N-demethylation of IMI only to a minor extent. In this regard, attempts were made to form DMI or 2-OH-IMI from IMI *in vitro* with two other commercially-available (Gentest 1992) human enzyme preparations (CYP1A1 and CYP3A4), but no DMI and 2-OH-IMI were detected with either enzyme system. Based on literature evidence, a member of another isozyme subfamily, CYP2C, may contribute to the N-demethylation of IMI (Skjelbo *et al.* 1991). However, since CYP2C isozyme was not readily available, a metabolic study of IMI with this isozyme could not be carried out in our laboratory.

The delay in the formation of 2-OH-DMI from IMI (Figure 2.16) initially suggested that the production of 2-OH-DMI required the prior formation of DMI. To test this hypothesis, DMI and 2-OH-IMI were separately metabolized under identical conditions by the CYP2D6 enzyme system. The yield of 2-OH-DMI from DMI was 4.5 times that recovered from 2-OH-IMI (Figure 2.17). Thus, 2-OH-DMI is formed metabolically from both DMI and 2-OH-IMI but the preferred pathway involving CYP2D6 proceeds *via* DMI. In the study by Birgersson *et al.* (1986) on the ability of a purified CYP2D6 preparation from human liver to ring-oxidize DMI to 2-OH-DMI and N-demethylate IMI to DMI, it was found that the rate of formation of 2-OH-DMI from DMI was 3.5 times faster than DMI's formation from IMI. This figure is in a good agreement with our result using human CYP2D6 expressed in a human cell line, for which the comparative value was 3.4 (Figure 2.17).

This investigation suggests that metabolic 2-hydroxylation of IMI in humans is mainly under the control of CYP2D6 while 2-hydroxylation of DMI and N-demethylation of IMI and 2-OH-IMI are partially catalyzed by CYP2D6.

## 2.5 REFERENCES

Abernethy DR, Greenblatt DJ, Shader RI. Imipramine and desipramine disposition in the elderly. *J Pharmacol Exp Ther* (1985) **232**: 183-188.

Aeppli L. Teratologische studien mit imipramine an ratte und kaninchen. *Arzneimittel Forsch* (1969) **19**: 1617-1640.

Ardrey RE, Allan AR, Bal TS, Joyce JR, Moffat AC. In: *Pharmaceutical Mass Spectra*. The Pharmaceutical Press, London, (1985) pp. 156, 294, 311.

Baker GB, Greenshaw AJ. Effects of long-term administration of antidepressants and neuroleptics on receptors in the central nervous system. *Cell Mol Neurobiol* (1989) **9**: 1-44.

Bakish D. Breaking through. *Can Pharm J* (1991) **124**: 299-306 & 316.

Bertilsson L, Henthorn TK, Sanz E, Tybring G, Säwe J, Villén T. Importance of genetic factors in the regulation of diazepam metabolism: Relationship to S-mephenytoin but not debrisoquine, hydroxylation phenotype. *Clin Pharmacol Ther* (1989) **45**: 348-355.

Bickel MH, Minder R. Metabolism and biliary excretion of the lipophilic drug molecules, imipramine and desmethylimipramine in the rat - I, experiments *in vivo* and with isolated perfused livers. *Biochem Pharmacol* (1970) **19**: 2425-2435.

Bigger JT, Giardina EGV, Perel JM, Kantor SJ, Glassman AH. Cardiac antiarrhythmic effect of imipramine hydrochloride. *N Engl J Med* (1977) **296**: 206-208.

Birgersson C, Morgan ET, Jörnvall H, von Bar C. Purification of a desmethylimipramine and debrisoquine hydroxylating cytochrome P-450 from human liver. *Biochem Pharmacol* (1986) **35**: 3165-3166.

Blackwell B, Currah J. The psychopharmacology of nocturnal enuresis. In: *Bladder Control and Enuresis* (Kolvin I, MacKeith RC & Meadow SR eds.), Spastic International Medical Publications, London, Philadelphia, (1973) pp. 231-257.

Brøsen K, Zeugin T, Meyer UA. Role of P450IID6, the target of the sparteine-debrisoquine oxidation polymorphism, in the metabolism of imipramine. *Clin Pharmacol Ther* (1991) **49**: 609-617.

Brøsen K, Gram LF. Quinidine inhibits the 2-hydroxylation of imipramine and desipramine but not the demethylation of imipramine. *Eur J Clin Pharmacol* (1989) **37**: 155-160.

Brøsen K, Gram LF. First-pass metabolism of imipramine and desipramine: impact of the sparteine oxidation phenotype. *Clin Pharmacol Ther* (1988) **43**: 400-406.

Brøsen K, Gram LF, Klysner R, Otton SV, Bech P, Bertilsson L. Steady-state concentrations of imipramine and its metabolites in relation to the sparteine/debrisoquine polymorphism. *Eur J Clin Pharmacol* (1986a) **30**: 679-684.

Brøsen K, Otton SV, Gram LF. Imipramine demethylation and hydroxylation: impact of the sparteine oxidation phenotype. *Clin Pharmacol Ther* (1986b) **40**: 543-549.

Cholerton S, Daly AK, Idle JR. The role of individual human cytochromes P450 in drug metabolism and clinical response. *Trends Pharmacol Sci* (1992) **13**: 346-352.

Ciraulo DA, Barnhill JG, Jaffe JH. Clinical pharmacokinetics of imipramine and desipramine in alcoholics and normal volunteers. *Clin Pharmacol Ther* (1988) **43**: 509-518.

Crammer JL, Scott B, Rolfe B. Metabolism of $^{14}$C-imipramine: II. urinary metabolites in man. *Psychopharmacologia (Berl.)* (1969) **15**: 207-225.

Crammer J, Scott B, Woods H, Rolfe B. Metabolism of $^{14}$C-imipramine. I. Excretion in the rat and in man. *Psychopharmacologia (Berl.)* (1968) **12**: 263-277.

Dayton PG, Israili ZH, Cunningham RF, Stiller R, Perel JM. The effects of lipids on the binding of imipramine and other drugs to serum proteins. In: *Phenothiazines and Structurally Related Drugs* (Usdin E & Eck H eds.), Elsevier, Amsterdam, (1980) pp. 185-188.

DeVane C, Jusko W. Plasma concentration monitoring of hydroxylated metabolites of imipramine and desipramine. *Drug Intell Clin Pharm* (1981) **15**: 263-266.

Dencker H, Dencker SJ, Green A, Nagy A. Intestinal absorption, demethylation, and enterohepatic circulation of imipramine. *Clin Pharmacol Ther* (1975) **19**: 584-586.

Eichelbaum M, Spannbrucker N, Sieincke B, Dengler HJ. Defective N-oxidation of sparteine in man: a new pharmacogenetic defect. *Eur J Clin Pharmacol* (1979) **16**: 183-187.

Folsom JP. In: *Physicians' Desk Reference, 15th edition.* Medical Economics Inc., Oradell. NJ, (1961) pp. 593-594.

Glassman AH, Perel JM. The clinical pharmacology of imipramine. *Arch Gen Psychiatry* (1973) **28**: 649-653.

Gram LF, Kragh-Sørensen P, Kristensen CB, Møller M, Pedersen OL,Thayssen P. Plasma level monitoring of antidepressants: theoretical basis and clinical application. In: *Advances in Biochemical Psychopharmacology, vol. 39, Frontiers in Biochemical and Pharmacological Research in Depression* (Usdin E, Åsberg M, Bertilsson L & Sjöqvist F eds.), Raven Press, New York, (1984) pp. 399-411.

Gram LF, Pedersen OL, Kristensen CB, Bjerre M, Kragh-Sørensen P. Drug level monitoring in psychopharmacology: usefulness and clinical problems, with special reference to tricyclic antidepressants. *Ther Drug Monit* (1982) **4**: 17-25.

Gram L, Sondergaard I, Christiansen J, Petersen G, Bech P, Reisby N, Ibsen I, Ortmann J, Nagy A, Dencker S, Jacobsen O, Krautwald O. Steady-state kinetics of imipramine in patients. *Psychopharmacology* (1977) **54**: 255-261.

Gram LF, Christiansen J. First-pass metabolism of imipramine in man. *Clin Pharmacol Ther* (1975) **17**: 555-563.

Greenblatt DJ, Divoll M, Abernethy DR, Harmatz JS, Shader RI. Antipyrine kinetics in the elderly: Prediction of age-related changes in benzodiazepine oxidizing capacity. *J Pharmacol Exp Ther* (1982) **220**: 120-126.

Greenblatt DJ, Divoll M, Harmatz JS, Shader RI. Oxazepam kinetics: Effects of age and sex. *J Pharmacol Exp Ther* (1980) **215**: 86-91.

Grünthal E. Untersuchungen über die besondere psychologische Wirkung des Thymolepticums Tofranil. *Psychiat Neurol* (Basel) (1958) **136**: 402-408.

Hammer W, Sjoqvist F. Plasma levels of monomethylated tricyclic antidepressants during treatment with imipramine-like compounds. *Life Sci* (1967) **6**: 1895-1903.

Herrmann B, Pulver R. Des stoffswechsel des psychopharmakons tofranil. *Arch Int Pharmacodyn Ther* (1960) **126**: 454-469.

Horwell DC. Antidepressants In: *Drugs in Central Nervous System Disorders, Clinical Pharmacology vol 2* (Horwell DC ed.), Marcel Dekker Inc., New York, (1985) pp. 71-121.

Hufford C, Capiton G, Clark A, Baker J. Metabolism of imipramine by microorganisms *J Pharm Sci* (1981) **70**: 151-155.

Hughes FN (ed.). In: *Compendium of Pharmaceutical Specialties, 2nd edition.* The Canadian Pharmaceutical Association Inc., Thorn Press, Toronto, (1963) pp. 440.

Hunt CM, Westerkam WR, Stave GM. Effect of age and gender on the activity of human hepatic CYP3A. *Biochem Pharmacol* (1992) **44**: 275-283.

Imaoka S, Fujita S, Funae Y. Age-dependent expression of cytochrome P-450s in rat liver. *Biochim Biophys Acta* (1991) **1097**: 187-192.

Judd CI, Ursillo RC. Absorption, distribution, excretion, and metabolism of antidepressants. In: *Antidepressants, Industrial Pharmacology vol. II* (Fielding S & Lal H eds.), Futura Publishing Company, New York, (1975) pp. 231- 265.

Kitanaka I, Ross RJ, Cutler NR, Zavadil AP, Potter WZ. Altered hydroxydesipramine concentrations in elderly depressed patients. *Clin Pharmacol Ther* (1982) **18**: 517-520.

Klerman GL, Cole J. Clinical pharmacology of imipramine and related antidepressant compounds. *Pharmacol Rev* (1965) **17**:101-141.

Kristiansen ES. Cardiac complications during treatment with imipramine (Tofranil). *Acta Psychiatr Neurol Scand* (1961) **36**: 427-441.

Krogh CME. In: *Compendium of Pharmaceuticals and Specialties, 27th edition.* (Krogh CME & Gillis MC eds.), The Canadian Pharmaceutical Association Inc., Thorn Press, Toronto, (1992) pp. 1159-1160.

Krüger R, Hölzl G, Kuss HJ, Schefold L. Comparison of the metabolism of the three antidepressants, amitriptyline, imipramine, and chlorimipramine *in vitro* in rat liver microsomes. *Psychopharmacology* (1986) **88**: 505-513.

Kuhn R. The Imipramine Story. In: *Discoveries in Biological Psychiatry* (Ayd F & Blackwell B eds.), Lippincott, Philadelphia, (1970) pp. 205-217 .

Kuhn R. Die Behandlung depressiver Zustände mit einem Iminodibenzylderivat (G 22355). *Schweiz Med Wschr.* (1957) **87**: 1135-1140.

Kvinesdal B, Molin J, Frøland A, Gram LF. Imipramine treatment of painful diabetic neuropathy. *JAMA* (1984) **251**: 1727-1730.

Lieberman JA, Cooper TB, Suckow RF, Steinberg H, Borenstein M, et al. Tricyclic antidepressant levels in chronic renal failure. *Clin Pharmacol Ther* (1985) **37**: 301-307.

Lydiard RB, Roy-Byrne PP, Ballenger JC. Recent advances in the psychopharmacological treatment of anxiety disorders. *Hosp Community Psychiatry* (1988) **39**: 1157-1165.

Mahgoub A, Idle JR, Dring LG, Lancaster R, Smith RL. Polymorphic hydroxylation of debrisoquine in man. *Lancet* (1977) **2**: 584-586.

Mattick RP, Andrews G, Hadzi-Pavlovic D, Christensen H. Treatment of panic and agoraphobia. An integrative review. *J Nerv Ment Dis* (1990) **178**: 567-576.

Maurer H, Pfleger K. Screening procedure for detection of antidepressants and their metabolites in urine using a computerized gas chromatographic-mass spectrometric technique. *J Chromatogr* (1984) **305**: 309-323.

Minder R, Schnetzer F, Bickel MH. Hepatic and extrahepatic metabolism of the psychotropic drugs, chlorpromazine, imipramine, and imipramine-N-oxide. *Naunyn-Schmiedebergs Arch Pharmacol* (1971) **268**: 334-347.

Muller OF, Goodman N, Bellet S. The hypotensive effect of imipramine hydrochloride in patients with cardiovascular disease. *Clin Pharmacol Ther* (1961) **2**: 300-307.

Nagy A, Treiber L. Quantitative determination of imipramine and desipramine in human blood plasma by direct densitometry of thin layer chromatograms. *J Pharm Pharmacol* (1973) **25**: 599-603.

Nagy A, Johansson R. Plasma levels of imipramine and desipramine in man after different routes of administration. *Naunyn-Schmiedebergs Arch Pharmacol* (1975) **290**: 145-160.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, Gonzalez FJ, Guengerich FP, Gunsalus IC, Johnson EF, Loper JC, Sato R, Waterman MR, Waxman DJ. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA Cell Biol* (1991) **10**: 1-14.

Nelson JC, Atillasoy E, Mazure C, Jatlow PI. Hydroxydesipramine in the elderly. *J Clin Psychopharmacol* (1988) **8**: 428-433.

Ochs HR, Greenblatt DJ, Divoll M, Abernathy DR, Feyerabend H, Dengler HJ. Diazepam kinetics in relation to age and sex. *Pharmacology* (1982) **14**: 341-345.

O'Malley K, Crooks J, Duke E, Stevenson IH. Effect of age and sex on human drug metabolism. *Br Med J* (1971) **3**: 607-609.

Potter WZ, Rudorfer MV, Elizabeth AL. Active metabolites of antidepressants: pharmacodynamics and relevant pharmacokinetics. In: *Frontiers in Biochemical and Pharmacological Research in Depression, Advances in Biochemical Psychopharmacology vol. 39.* (Usdin E, Åsberg M, Bertilsson L & Sjöqvist F eds.), Raven Press, New York, (1984), pp. 373-390.

Potter WZ, Calil HM. Metabolites of tricyclic antidepressants: biological activity and clinical implications. In: *Clinical Pharmacology in Psychiatry* (Usdin E ed.) Elsevier, New York, (1981) pp. 311-324.

Pscheidt GR. Demethylation of imipramine in male and female rats. *Biochem Pharmacol* (1962) **11**: 501-501.

Rubinstein G, McIntyre I, Burrows GD, Norman TR, Maguire KP. Metabolism of tricyclic antidepresant drugs. In: *Antidepressants* (Burrows GD, Norman TR & Davies B eds), Elsevier, Amsterdam, (1983) pp. 57-74,

Sallee FR, Pollock BG. Clinical pharmacokinetics of imipramine and desipramine. *Clin Pharmacokinet* (1990) **18**: 346-364.

Sarwer-Foner GJ, Grauer H, Mackay J, Koranyi EK. Depressive states and drugs -A study of the use of imipramine "Tofranil" in open psychiatric settings. *Can Med Serv J* (1959) **15:** 359-382.

Schindler W, Hafliger F. Derivatives of iminodibenzyl. *Helv Chim Acta* (1954) **37**: 472-483.

Skjelbo E, Brøsen K. Inhibitors of imipramine metabolism by human liver microsomes. *Br J Clin Pharmacol* (1992) **34**: 256-261.

Skjelbo E, Brøsen K, Hallas J, Gram LF. The mephenytoin oxidation polymorphism is partially responsible for the N-demethylation of imipramine. *Clin Pharmacol Ther* (1991) **49**: 18-23.

Sloman L. Mycocardial infarction during imipramine treatment of depression. *Can Med Ass J* (1960) **82**: 20-22.

Smith DA. Species differences in metabolism and pharmacokinetics: are we close to an understanding? *Drug Metab Rev* (1991) **23**: 355-373.

Sovner R, Orsulak P. Excretion of imipramine and desipramine in human breast milk. *Am J Psychiatry* (1979) **136**: 451-452.

Spina E, Steiner E, Ericsson Ö, Sjöquist F. Hydroxylation of desmethylimipramine: dependence on the debrisoquine hydroxylation phenotype. *Clin Pharmacol Ther* (1987) **41**: 314-319.

Spina E, Birgersson C, von Bahr C, Ericsson Ö, Mellström, Steiner E, Sjöqvist F. Phenotypic consistency in hydroxylation of desmethylimipramine and debrisoquine in healthy subjects and in human liver microsomes. *Clin Pharmacol Ther* (1984) **36**: 677-682.

Stahl SM, Palazidou L. The pharmacology of depression: studies of neurotransmitter receptors lead the search for biochemical lesions and new drug therapies. *Trends Pharmacol Sci* (1986) **7**: 349-354.

Steiner E, Dumont E, Spina E, Dahlqvist R. Inhibition of desipramine 2-hydroxylation by quinidine and quinine. *Clin Pharmacol Ther* (1988) **43**: 577-581.

Sutfin TA, DeVane CL, Jusko WJ. The analysis and disposition of imipramine and its active metabolites in man. *Psychopharmacology* (1984) **82**: 310-317.

Svensson TH. Central alpha-adrenoceptors and the mechanisms of action of antidepressant drugs. In: *Advances in Biochemical Psychopharmacology, vol. 39, Frontiers in Biochemical and Pharmacological Research in Depression.* (Usdin E, Åsberg M, Bertilsson L & Sjöqvist eds.), Raven Press, New York, (1984) pp. 241-248.

Torok-Both G. Analysis of phenelzine, lidocaine and doxapram. (Thesis submitted to Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta), (1987).

von Bahr C, Guengerich FP, Movin G, Nordin C. The use of human liver banks in pharmacogenetic research. In: *Clinical Pharmacology in Psychiatry, Psychopharmacology, series 7* (Dahl SG & Gram LF eds.), Springer-Verlag, Berlin, (1989) pp. 163-171.

von Bahr C, Spina E, Birgerson C, Ericsson O, Goransson M, Henthorn T, Sjöqvist F. Inhibition of desmethylimipramine 2-hydroxylation by drugs in human liver microsomes. *Biochem Pharmacol* (1985) **34**: 2501-2505.

Weder HJ, Bickel MH. Separation and determination of imipramine and its metabolites from biological samples by gas-liquid chromatography. *J Chromatogr* (1968) **37**: 181-189.

Wilson K. Sex-related differences in drug disposition in man. *Clin Pharmcokinet* (1984) **9**: 189-202.

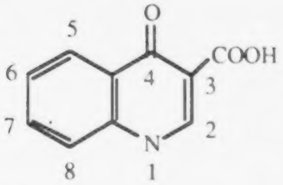
Zeidenberg P, Perel JM, Kanzler M, Warthon RN, Malitz S. Clinical and metabolic studies with imipramine in man. *Am J Psychiatry* (1971) **127**: 1321-1326.

Zeugin TB, Brosen K, Meyer UA. Determination of imipramine and seven of its metabolites in human liver microsomes by a high-performance liquid chromatographic method. *Anal Biochem* (1990) **189**: 99-102.

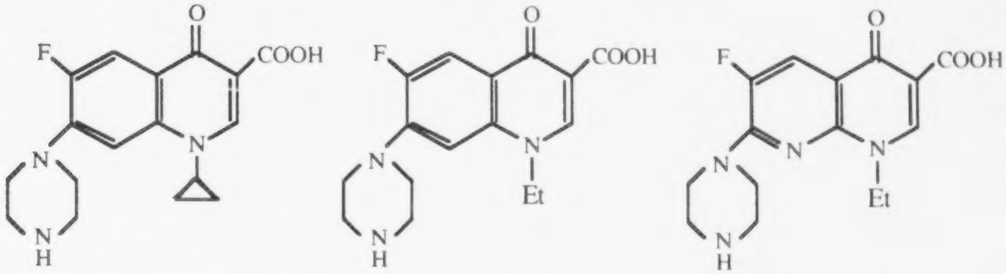
# Chapter 3. PEFLOXACIN

## 3.1 INTRODUCTION

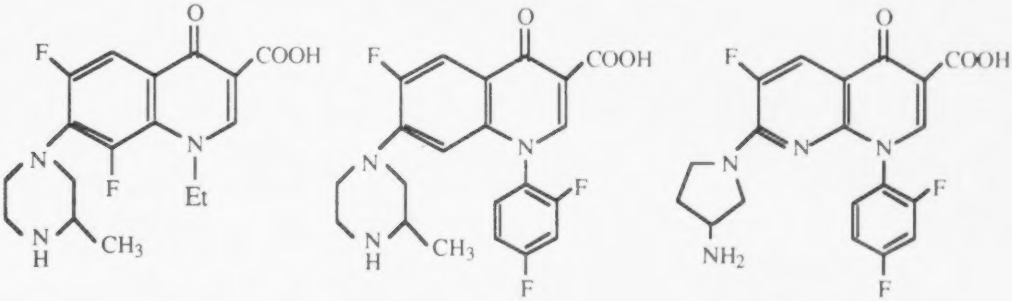
Fluoroquinolones are a new class of antibacterial agents developed in 1980. Structurally, they are related to nalidixic acid, an agent used in the 1960s to treat urinary tract infections. Structure modifications of nalidixic acid by introducing a fluorine atom at the 6 position and piperazine substituents at the 7 position on the quinolone nucleus produced a new generation of quinolones named fluoroquinolones (Figure 3.1). Compared to the prototype, fluoroquinolones possess much higher potency (1000 times), a broader spectrum of activity, lower minimum inhibitory concentrations (MIC), and improved oral bioavailability and tissue penetration (Fitton 1992). Their antibacterial activity, pharmacology and clinical implications have been extensively reviewed by many authors (Dabbs *et al.* 1987; Hooper & Wolfson 1985, 1991; Wolfson & Hooper 1985, 1989; Andriole 1988; Neu 1988; Neuman 1988; Felmingham 1989; Fitton 1992). There were six fluoroquinolones marketed in USA, but one of them (temafloxacin; *Omniflox*) was recently withdrawn because of reports of a high incidence of hemolysis and renal dysfunction. The remaining five are norfloxacin (*Noroxin*), marketed only for urinary tract infections; lomefloxacin (*Maxaquin*) for urinary tract infections, bronchitis and prophylaxis before transurethral surgical procedures; enoxacin (*Penetrex*), available for oral use for treatment of urinary tract infections and uncomplicated urethral or cervical gonorrhea; and ciprofloxacin (*Cipro*) and ofloxacin (*Floxin*), available for both oral and parenteral use in many indications (Abramowicz 1992). Other fluoroquinolones available in Japan and countries in Europe include pefloxacin and tosufloxacin (Figure 3.1).


O
COOH
5
6
7
8
4
3
2
1
N

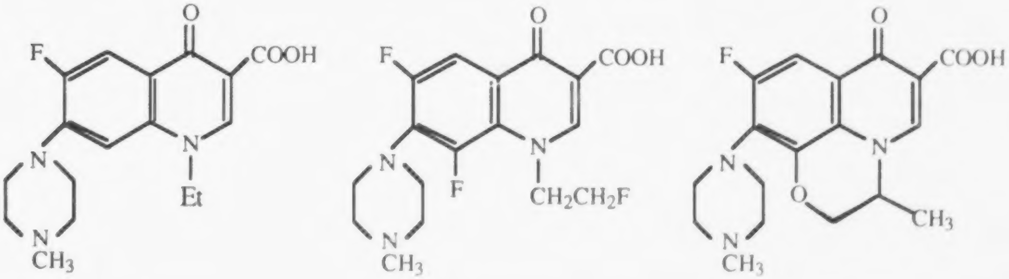
Quinolone nucleus


O
F
COOH
N
N
H
Et

Ciprofloxacin Norfloxacin Enoxacin


O
F
COOH
N
H
CH3
Et
NH2

Lomefloxacin Temafloxacin Tosufloxacin

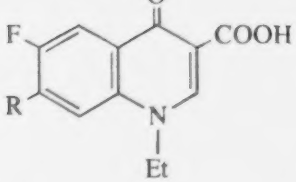

O
F
COOH
N
CH3
Et
CH2CH2F
O

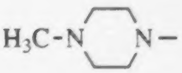
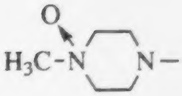
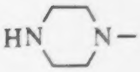
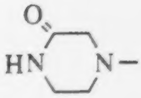
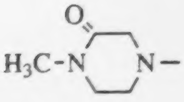
Pefloxacin Fleroxacin Ofloxacin

**Figure 3.1 Structures of some fluoroquinolones**

Drug interactions of fluoroquinolones with other agents that have the potential for clinical disturbances have been documented (Wolfson & Hooper 1989; Prince 1989; Davey 1988). One important interaction is that between fluoroquinolones and xanthine derivatives such as theophylline. Symptoms of theophylline toxicity have been reported in elderly patients 4 to 5 days after coadministration of ciprofloxacin and theophylline (Holden 1988). The degree of inhibition of theophylline clearance seems to be partly related to the extent of the formation of the oxo-metabolite of quinolones (Figure 3.2). Thus, ofloxacin, which undergoes least metabolism has little effect whereas ciprofloxacin, pefloxacin and enoxacin, which are metabolized to a greater extent have an intermediate to a marked effect. The exact mechanism involved is still not clear, although it has been suggested that the interaction is produced through an effect on the cytochrome P450 isozymes that catalyze the N-demethylation of theophylline (Wijnands & Vree 1988).

Among the fluoroquinolones, drug metabolism occurs to the greatest extent with pefloxacin. Most of the reported methods for the estimation of the extent of metabolism of fluoroquinolones are based on HPLC analysis. No GC method has been reported in literature. It was of interest to examine the metabolism pathway of pefloxacin *in vitro* with purified human cytochrome P450 isozymes and to investigate the involvement of these isozymes in its metabolic N-demethylation, thereby providing information helpful for understanding of drug interactions involving pefloxacin. This interest in the metabolism of pefloxacin required the development of a gas chromatographic procedure which could be coupled to a mass spectrometer for the identification of novel metabolites.



| R | Designation | Recovered in urine after administration of pefloxacin (% of dose)* |
|---|---|---|
|  | Pefloxacin | 9% |
|  | Pefloxacin N-oxide | 23% |
|  | Norfloxacin | 20% |
|  | 3-Oxo-norfloxacin | 5% |
|  | 3-Oxo-pefloxacin | trace |

* 60% of the administered drug recovered in human urine as free drug and the above metabolites (Montay *et al.* 1984).

**Figure 3.2 Structures of pefloxacin and its four metabolites.**

## 3.1.1 Pefloxacin

### 3.1.1.1 Chemistry

Pefloxacin (1589 RB) was first synthesized by Goueffon *et al.* in 1981 in Laboratoire Roger Bellon, France, and marketed under the trade name of *Peflacine* (pefloxacin mesylate dihydrate, Figure 3.3) for oral and parenteral use in France in 1985. The chemical name of pefloxacin is 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(4-methyl-1-piperazinyl)-quinoline-3-carboxylic acid. The piperazinyl substituent at the 7 position gives pefloxacin amphoteric ionic properties. The pKa values corresponding to the carboxylic function and to the basic amine of 4-methyl-1-piperazinyl substituent are 6.3 and 7.6, respectively (Bergogne-Bérézin 1991). The dissociation of pefloxacin is illustrated in Figure 3.4.

### 3.1.1.2 Microbiology

#### *3.1.1.2.1 Antibacterial activity*

Pefloxacin possesses a broad spectrum of antimicrobial activity. It is a potent inhibitor of gram-negative bacteria, including most of Enterobacteriaceae ($MIC_{90} \leq 2$ mg/l), *Neisseria gonorrhoeae* ($MIC_{90} \leq 0.06$ mg/l) and *N. meningitidis* ($MIC_{90} \leq 0.06$ mg/l), *Haemophilus influenzae* ($MIC_{90}$ 0.05 to 0.5 mg/l) and *Pseudomonas aeruginosa* ($MIC_{90}$ 2 to 16 mg/l). Pefloxacin is also active against some gram-positive bacteria such as *Staphylococcus aureus*, including those resistant to penicillin, oxacillin and methicillin ($MIC_{90}$ 0.25 to 3.8 mg/l). Other gram-positive organisms such as *Mycobacterium tuberculosis* were moderately susceptible to pefloxacin ($MIC_{90}$ 0.3 to 2.5 mg/l) and *Streptococcus* species were moderately susceptible or resistant to pefloxacin ($MIC_{90}$ 3.1 to 32 mg/l). The activity of pefloxacin against intracellular bacteria such as *Chlamydia*,

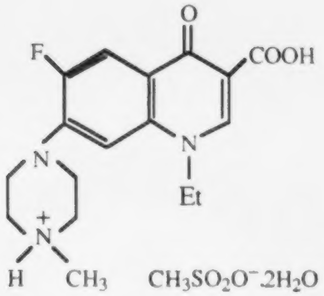

O
F
COOH
N
N
Et
+
N
H
CH3
CH3SO2O−.2H2O

Figure 3.3 Structure of pefloxacin mesylate dihydrate.

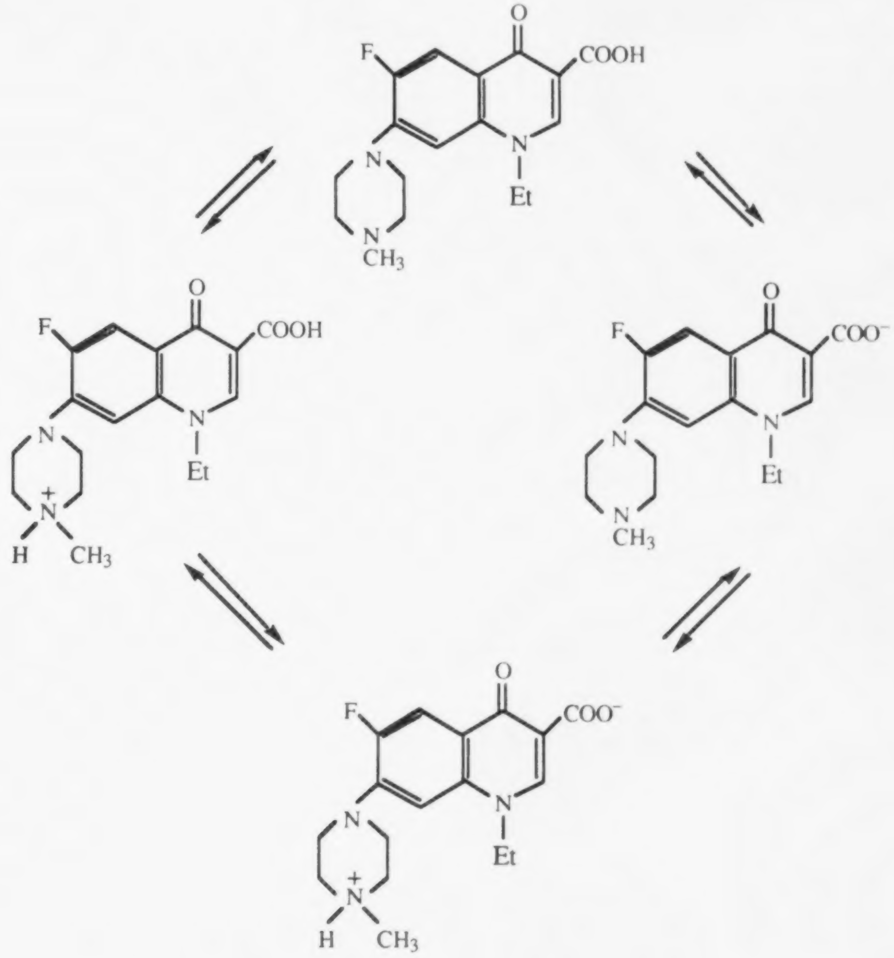

O
F
COOH
COO−
N
Et
+
H
CH3

Figure 3.4 The dissociation of pefloxacin.

*Mycoplasma* and *Ureaplasma* was generally low, except for moderate activities against *Ureaplasma urealyticum* ($MIC_{90}$ 2 to 8 mg/l) and *Chlamydia trachomatis* ($MIC_{90}$ 8 mg/l) (Gonzalez & Henwood 1989).

The *in vitro* antibacterial activity of pefloxacin was found to be influenced by the pH of the growth media. In acidic solution (pH 5.5) the MIC value increased 8-fold over that at pH 7.4 (Neu *et al.* 1984). This may be due to the quaternarization of the 7-piperazinyl moiety, which causes interference with cell penetration or interaction with the intracellular target (Smith & Ratcliffe 1986). Supplementation with calcium or magnesium ions reduced the activity of pefloxacin (Auckenthaler *et al.* 1986). Minimum bactericidal concentration (MBC) values of pefloxacin are similar to the MIC values (MBC = 1-2 MIC) against both gram-negative and gram-positive organisms.

### *3.1.1.2.2 Mode of action*

Like other members of the fluoroquinolone series, the primary target of pefloxacin is DNA gyrase, an enzyme essential for bacterial DNA replication and repair. This enzyme is a topoisomerase II and is composed of two A subunits and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. DNA gyrase is responsible for introducing negative supercoil into covalently closed circular DNA and separating interlocked DNA molecules. These processes require the energy from ATP hydrolysis and involve breakage and rejoining of both strands of DNA. Pefloxacin inhibits the DNA synthesis by blocking the rejoining step. This primary mechanism is supported by the observation that mutational alterations in the A or B subunit result in bacterial resistance to fluoroquinolones (Lewin *et al.* 1990; Piddock *et al.* 1989), and by the finding of a good correlation between the MICs and the inhibition of gyrase-dependent DNA supercoiling

(Ronnlund *et al.* 1985; Sato *et al.* 1985). Even though many details of the molecular interactions between DNA gyrase, DNA and fluoroquinolones have not been elucidated, three models of fluoroquinolone binding sites have been suggested: direct binding to DNA (Shen & Pernet 1985), binding to a complex of gyrase and DNA (Shen *et al.* 1989), and binding directly to gyrase only (LeGoffic 1985).

Although there is usually a correlation between the antibacterial activity and the inhibition of DNA synthesis of quinolones, it is not considered to be the only mechanism for the effectiveness of quinolones. It has been shown that synthesis of bacterial protein and RNA as well as bacterial division are essential for the demonstration of the antibacterial activity of quinolones (Smith 1984; Smith & Lewin 1988; Dietz *et al.* 1966). Smith & Lewin (1988) proposed three bactericidal mechanisms for quinolones against *E coli* KL16. Mechanism A applies to all quinolones. This mechanism is the principal bactericidal one and requires the processes of cell division and syntheses of protein and RNA to be actively occurring. This is based on the observation that addition of rifampicin (an inhibitor of RNA synthesis) or chloramphenicol (an inhibitor of protein synthesis), or transfer of bacterial cells into phosphate-buffered saline (a medium in which bacterial division cannot take place) completely abolished the bactericidal activity of nalidixic acid. In addition to mechanism A, an unknown secondary mechanism termed B is suggested for ciprofloxacin, ofloxacin, pefloxacin, lomefloxacin and fleroxacin (Smith 1984; Lewin *et al* 1989; Lewin & Amyes 1990). Mechanism B does not require protein or RNA synthesis and applies to non-dividing bacteria. Another unknown secondary mechanism named C is exerted by norfloxacin and enoxacin. Mechanism C requires syntheses of protein and RNA, and also applies to the non-dividing bacteria. The possession of a secondary bactericidal mechanism by fluoroquinolones in addition to the primary mechanism explains why these drugs are more active than nalidixic acid.

#### *3.1.1.2.3 Development of resistance*

The frequency of resistance to pefloxacin is extremely low ($<10^{-8}$) (Gonzalez & Henwood 1989). The resistance develops *via* chromosomal mutation of DNA gyrase and outer membrane protein (Neuman 1988). The first one (Hooper *et al.* 1987; Kayser 1985) confers resistance to quinolones only, while the latter (Hooper *et al.* 1987; Piddock *et al.* 1987) confers cross-resistance. Plasmid-mediated resistance to pefloxacin does not occur and this is a unique characteristic of pefloxacin among the quinolones. Lack of the plasmid-mediated transferable resistance might be the reason that quinolones have a relatively low frequency of resistance.

### 3.1.1.3 Pharmacokinetics

Pharmacological properties of pefloxacin following oral and intravenous administration have been studied in healthy volunteers. Following single oral doses of 400 mg pefloxacin, the peak serum concentration (4.3 mg/l) was attained at about 1.5 h. The area under the plasma concentration-time curve (AUC) values are virtually identical when the intravenous and oral routes of administration of single 400 mg doses are compared (Barre *et al.* 1984; Sörgel *et al.* 1988a). Thus, the bioavailability of oral pefloxacin is considered as 100%, or complete. Pefloxacin binding to human serum proteins is approximately 20 to 30%, a relatively low value, which is a characteristic of piperazinyl-substituted fluoroquinolones (Montay *et al.* 1984; Neuman 1987). The elimination half-life of pefloxacin in plasma ranges from 7.2 to 13 h after administration of a single oral dose of 400 mg (Barre *et al.* 1984; Höffler *et al.* 1988; Webberley *et al.* 1987). Pefloxacin undergoes extensive metabolism (85-90%). As one would expect, the pharmacokinetics of pefloxacin are influenced by hepatic function. In a study conducted by Danan *et al.* (1985), the elimination half-life of pefloxacin was significantly longer in 16 patients with liver cirrhosis (35 h) than in the 12 healthy subjects (11 h), suggesting the requirement of dosage adjustments in treating patients with liver disease. The elimination half-life of pefloxacin is not altered in patients with impaired renal function. Montay *et al.* (1985a) found that the pefloxacin elimination half-life was not significantly different in normal renal function (6-14 h) compared with that in renal failure (10-15 h).

Pefloxacin appears to distribute widely in body fluids, with a high volume of distribution ($V_d$=65 L). Relative to its plasma concentration, pefloxacin achieves equivalent or higher levels in aortic valve, blister fluid, bone, cardiac muscle, mitral valve, peritoneum, prostate gland, saliva and sputum (Gonzalez & Henwood 1989). In brain and cerebrospinal fluid pefloxacin concentrations are lower than in plasma, but are higher than MIC values (Gonzalez & Henwood 1989). Pefloxacin concentration in urine, feces, kidney and prostatic tissue suggest its usefulness for the treatment of urinary tract infections, prostatitis, gonorrhea and bacterial gastroenteritis. In addition, pefloxacin concentrations achieved in blood, lung, bone, and other body tissues and fluids often exceed its MIC for gram-negative bacteria and certain gram-positive cocci, indicating its efficacy in the treatment of systemic infections.

### 3.1.1.4 Clinical trials

Most clinical trials with oral or intravenous pefloxacin have been conducted in hospitalized patients with a variety of infectious diseases.

Pefloxacin has been shown to achieve concentrations in bronchial secretions approaching those in serum. In 46 patients with nosocomially acquired pulmonary infections, pefloxacin (400 mg 2 or 3 times daily for 4 to 28 days) produced a clinical cure in 67% of cases, with bacteriological eradication of 85% of isolated pathogens (Martin *et al.* 1988). Resistant strains of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* were responsible for some of the treatment failures. In the treatment of severe lower respiratory tract infections, Vargas *et al.* (1987) reported that oral administration of pefloxacin (400 mg 2 or 3 times daily for 10 days) produced 81% clinical cure and 95% bacteriological cure. Pefloxacin (800 to 1200 mg daily) was administered to patients with gynecological infections, complicated upper and lower urinary tract infections, and pelvic infections associated with the genital tract. An overall clinical cure of 72 to 94% was obtained in a patient population of 88 and 15, respectively (Boerema 1986; Peixoto 1987).

Pefloxacin showed a good penetration into bone tissue, achieving concentrations of at least 1 mg/kg, high enough to inhibit staphylococcal strains (Dellamonica *et al.* 1986). In the treatment of bone and joint infections, oral pefloxacin 400 mg twice daily for up to 18 months gave a clinical cure in 86 to 100% of patients. In 48 patients with chronic osteomyelitis, treatment with pefloxacin (400 mg twice daily) produced a clinical cure in 89.6% of patients (Desplaces *et al.* 1985). Pefloxacin has also been used successfully for the treatment of fever or infections in a limited number of immunologically compromised patients (Beun *et al.* 1988; Segev *et al.* 1987).

In a randomized comparative study of 42 patients with typhoid fever, pefloxacin (800 mg daily) was as effective as co-trimoxazole (trimethoprim 320 mg; sulfamethoxazole 1600 mg) in a 2-week treatment. Both drugs produced a clinical cure in 100% of patients, but pefloxacin gave a more rapid onset of apyrexia than co-trimoxazole, allowing a shortening of the treatment time (Hajji *et al*. 1988).

#### *3.1.1.4.1 Side effects*

Pefloxacin is generally well tolerated. The most common adverse effects are related to the gastrointestinal tract. In a survey of 1437 patients, over 50% of them reported experiencing gastrointestinal disturbances (Gonzalez & Henwood, 1989). Nausea was the most frequently cited adverse effect. The incidence of photosensitivity was 0.83% of patients in this survey after exposure to sunlight during therapy.

### 3.1.2 Metabolism

Among fluoroquinolones, pefloxacin undergoes the greatest extent of biotransformation (85 to 90%) (Gonzalez & Henwood 1989). The major metabolic pathways of pefloxacin are N-demethylation to form norfloxacin and N-oxidation to form pefloxacin N-oxide (Figure 3.2). Formations of oxo-norfloxacin and oxo-pefloxacin are considered as minor pathways. The 4-methylpiperazinyl substituent at position 7 of pefloxacin is the most prominent site where metabolism occurs (Figure 3.2). N-Oxidation, N-demethylation and oxo-formation of pefloxacin all occur at this site. Unlike other fluoroquinolones such as norfloxacin (agent with a piperazinyl substitution at position 7) where a 2-aminoethylamino-metabolite is formed by piperazinyl ring cleavage to —$NHCH_2CH_2NH_2$, the extent of the piperazinyl ring cleavage of pefloxacin is negligible (Sörgel 1989). A similar situation was found in the formation of oxo-metabolites. The formation of the oxo-metabolite is greatest for enoxacin (11% of dose) and is lowest for pefloxacin (0.75%) where the oxo-formation may be affected by steric hindrance of the methyl group (Sörgel 1989). The N-oxide was

formed only in agents with a methyl substituent at the 4 position of the piperazine substituent such as pefloxacin. In the rat model, the N-oxide has been found to be rebiotransformed into its parent compound (Sörgel 1989).

It has been shown that N-oxide formation from tertiary aliphatic amines is catalyzed by microsomal flavin-containing monooxygenases (Ziegler 1980), while N-demethylation and oxo-formation are catalyzed by cytochrome P450 isozymes since these latter two metabolic pathways are inhibited by cimetidine and SKF 525A (Mahr *et al.* 1989).

Montay *et al.* (1984) investigated the metabolism of pefloxacin in mice, rats, dogs, monkeys and humans. In humans, after a single oral dose of 800 mg, the total urinary recovery of the parent drug plus metabolites accounted for 60% of dose of which 20% was norfloxacin, 23% was pefloxacin N-oxide, 5% was oxo-norfloxacin and 9% was pefloxacin. Trace quantities of pefloxacin glucuronide were also detectable in urine. Some of the remaining 40% may be excreted in feces. In fact, approximately 20% of the radioactivity was detected in feces within 72h following administration of a single oral dose of $^{14}$C-pefloxacin (Gonzalez & Henwood 1989). Little information is currently available on the excretion of pefloxacin and its metabolites in human bile, although biliary excretion of pefloxacin as parent drug and glucuronide conjugate has been documented (Montay *et al.* 1984; Roy *et al.* 1987). After administration of 400 mg of pefloxacin to eight healthy volunteers, the 96 h urinary recoveries of pefloxacin N-oxide, norfloxacin and pefloxacin were 17.9%, 16.6% and 7.6%, respectively (Sörgel *et al.* 1988b).

The factors affecting the metabolism of fluoroquinolones have not been investigated in detail. Smoking seems to induce N-oxidation and N-demethylation and decrease the elimination half-life of pefloxacin (Jaehde *et al.* 1987).

There is species variation in the metabolism of pefloxacin (Montay *et al.* 1984). In mice, the urinary recovery of identified metabolites (glucuronide and N-oxide) was 29.5%. Urinary recoveries of the major metabolites (glucuronide, N-oxide and norfloxacin) and parent drug in rat and dog were similar (37.8% and 36.3%, respectively). Metabolites detected in monkey urine were norfloxacin, small amounts of pefloxacin N-oxide, oxo-pefloxacin, oxo-norfloxacin and traces of pefloxacin glucuronide, together with parent drug consisting of 26.5% of the administered dose. In human, the total urinary recovery of pefloxacin and metabolites accounted for 58.9% of the dose of drug. Metabolites included norfloxacin, pefloxacin N-oxide, oxo-norfloxacin, traces of oxo-pefloxacin and pefloxacin glucuronide; there also was some intact pefloxacin. Biliary excretion was extensive in rats (35% of dose) and dogs (20%), and less extensive in monkeys and humans (data not shown, Montay *et al.* 1983). It is noteworthy that no oxo-metabolite was detected in rat or dog, suggesting a lack of the enzyme which catalyzes the formation of this metabolite in these two species. It has been suggested that oxo-metabolites are involved in the drug interaction of quinolone with theophylline by inhibiting N-demethylation of theophylline (Wijnands & Vree 1988). In summary, glucuronidation of pefloxacin was found to be highest in rats and dogs and lowest in humans and monkeys. Pefloxacin N-oxide formation was shown to be the highest in humans, followed by dogs and rats. Oxo-metabolites were formed in humans and monkeys, but were not detectable in rats, mice and dogs (Montay *et al.* 1983).

An *in vitro* metabolic study of pefloxacin has been carried out in isolated perfused rat liver (Metz *et al.* 1989). The major metabolites detected were N-desmethylpefloxacin (2% of dose), pefloxacin N-oxide (7%) and an acyl-glucuronide of pefloxacin (20%) which is new metabolite found only in this metabolic system. In the same study, rebiotransformation of pefloxacin N-oxide to its parent compound was confirmed by adding pure pefloxacin N-oxide to the perfusion system. The metabolic conversion of pefloxacin N-oxide to pefloxacin was determined to be 30% of the dose. *In vitro* metabolism of pefloxacin by hepatic microsomes from different species (rats, mice, pigs and humans) was investigated by Mahr *et al.* (1989). Studies on potential inhibitors of pefloxacin N-demethylation in rat liver microsomes indicated that the demethylation of pefloxacin was strongly inhibited by SKF 525A and by cimetidine, suggesting that this metabolic pathway was catalyzed by cytochrome P450.

### 3.1.2.1 Drug interactions

#### *3.1.2.1.1 Inhibition of metabolism of methylxanthines by quinolones*

One of the important drug interactions of fluoroquinolones is that with xanthine derivatives such as theophylline. Enoxacin was first reported to interact with theophylline. Clinical theophylline toxicity including severe nausea (80% incidence), seizure (6.7%) and hallucination (6.7%) were reported in 15 patients who suffered with lower respiratory tract infections and were given enoxacin and theophylline concurrently (Maesen *et al.* 1984). In one study, an increase of 19.6% in plasma theophylline concentration and a 29.4% decrease in total body clearance were reported following coadministration of pefloxacin (Wijinands *et al.* 1986). The mechanism of this interaction is not yet understood. It was first suggested that the observed interaction was caused by the oxo-metabolite rather than by the parent drug (Wijinands *et al.* 1986) since the degree of inhibition seemed to correlate well with the degree of the production of the oxo-metabolite. However, a subsequent confirmative study on the inhibition of theophylline metabolism in rat liver microsomes by enoxacin and its metabolite, oxo-enoxacin, rejected this hypothesis, since the results of the study showed that enoxacin inhibited the formation of 1,3-dimethyluric acid from theophylline by 67% at 1.0 mM while oxo-enoxacin had no inhibitory effect (Mulder *et al.* 1988). It is now accepted that oxo-formation is not involved in the inhibition of theophylline metabolism.

Many research groups have tried to understand the mechanism of this drug-drug interaction from different aspects. Inhibitory potencies of quinolones against the cytochrome P450 isozyme CYP1A2 *in vitro* and *in vivo* were determined in human by Fuhr *et al.* (1992) using 3-demethylation rate of caffeine to measure the CYP1A2 activity; pefloxacin caused 22.0% reduction in CYP1A2 activity *in vitro* while enoxacin showed the highest inhibitory potency (74.9%). Somewhat surprisingly, ofloxacin and lomefloxacin showed 11.8% and 23.4% reduction, respectively, which is in disagreement with *in vivo* results where ofloxacin and lomefoxacin have no effect on theophylline and caffeine clearance (Wijnands *et al.* 1986; Harder *et al.* 1988; Robson 1992).

Robson (1992) suggested that those quinolones that have strong inhibitory potencies on theophylline clearance are stereochemically similar to theophylline. Modifications at position 8 of quinolone nucleus (such as in ofloxacin and lomefloxacin) would result in steric hindrance and decrease the structural similarity to theophylline. A recent study (Fuhr *et al.* 1993) on the relationship between structure and *in vitro* inhibition of the human cytochrome P450 isozyme CYP1A2 by 44 quinolones and derivatives suggested that the core of the molecule (naphthyridine or quinoline) and substituents at position 1 had pronounced effects on the CYP1A2 inhibitory potency, with naphthyridine as the molecular core and a cyclopropyl group at position 1 exhibiting the strongest inhibitory effect. A study of the effects of various substituents at position 7 of the quinolone on the inhibition of CYP1A2 activities indicated that the products of ring cleavage [piperazinyl ring → $-NH-(CH_2)_2-NH_2$] showed marked inhibitory effects on CYP1A2 activities. Reduced inhibitory potencies were observed in quinolones that possessed a piperazinyl ring, a methylated or ethylated piperazinyl ring, and a 3'-oxo-piperazinyl ring at the 7 position of the quinolone nucleus (Figure 3.1). Substitution with a fluoro or methyl group at the 7 position of the quinolone nucleus also resulted in strong inhibitory effects on CYP1A2. Derivatives with a fluoro substituent at position 8 were weak inhibitors. Based on molecular modeling, the 4-oxo-3-carboxylic acid moiety of the quinolone and the core of the molecule itself are believed to be involved in binding to CYP1A2. This study (Fuhr *et al.* 1993) showed that the *in vitro* potency of quinolones to inhibit CYP1A2 activity was directly dependent on their molecular structures. It was concluded that the inhibitory potency of a quinolone *in vivo* may actually be a reflection of the sum of the inhibitory effects of both the quinolone compound and its metabolites.

#### *3.1.2.1.2 Inhibition of metabolism of other drugs by quinolones*

It has been claimed that enoxacin and ciprofloxacin impair the clearance of antipyrine in man (Davey 1988). This suggested that ciprofloxacin and enoxacin inhibit more than one CYP isozyme since antipyrine and theophylline are known to be metabolized by different CYP isozymes (Gibson & Skett 1986). Metabolism of chlorpropamide, glibenclamide, cyclosporin A and erythromycin are not affected by quinolones such as enoxacin, ciprofloxacin and pefloxacin (Davey 1988; Pichard *et al.* 1990; Sarkar *et al.* 1990), indicating that quinolones are selective inhibitors of cytochrome P450s.

### 3.1.2.2 Identifying the CYP isozyme involved in N-demethylation of pefloxacin

The enzyme directly involved in N-demethylation of pefloxacin is not yet known. The fact that pefloxacin competitively inhibits 3-demethylation of caffeine may suggest that CYP1A2 is involved in N-demethylation of pefloxacin, or that pefloxacin and its metabolites simply bind to CYP1A2 and act as inhibitors of the isozyme. Pharmacokinetic interactions between theophylline and other drugs reveal that its clearance was decreased by enoxacin, ciprofloxacin, pefloxacin, propranolol, erythromycin, troleandomycin, verapamil, diltiazem and nifedipine (Fuhr *et al.* 1992; Upton 1991). It is known that the N-dealkylation of the last five compounds is catalyzed by CYP3A4. Since pefloxacin showed the same inhibitory effect on theophylline as these five compounds, one may expect that N-demethylation of pefloxacin to norfloxacin may also be catalyzed by CYP3A4. However, in a study of drug interactions with cyclosporin A, Pichard *et al.* (1990) reported that pefloxacin did not interfere with the *in vitro* human hepatic metabolism of cyclosporin A (which is a known substrate of CYP3A4). Surprisingly, quinidine, another known substrate of CYP3A4, was reported to have no effect on the metabolism of cyclosporine A in the same study. The results of this study also rule out the possibility that pefloxacin and other quinolones are nonspecific inhibitors of cytochrome P450 isozymes. Another CYP isozyme which is frequently involved in N-dealkylation is CYP2B4. In a rat hepatic microsomal preparation, ofloxacin, enoxacin and norfloxacin showed no significant effect on the activity of benzphetamine N-demethylase (Okazaki *et al.*

1988), suggesting that quinolone metabolism is not catalyzed by CYP2B4 [which is known to be involved in benzphetamine N-demethylation (Rodrigues *et al.* 1991)].

### 3.1.3 Analytical method

Most reported methods for analysis of quinolones involve HPLC. Various quinolones and their metabolites in biological samples such as blood, urine, bile, saliva, sputum and blister are analyzed using this technique (Vallée *et al.* 1986; Lebel *et al.* 1986; Jehl *et al.* 1985).

As shown in Figure 3.3, pefloxacin is an amphoteric compound with two ionizable groups (pKa 6.3 for the carboxyl group and 7.6 for the amine function). In most studies, plasma and urine concentrations of unchanged pefloxacin and its metabolites were measured by HPLC assays. Some methods are summarized in Table 3.1. Most quinolones and their metabolites are strongly fluorescent; therefore, HPLC with fluorometric detection allows higher analytical sensitivity than with UV detection.

The amphoteric properties of pefloxacin and other quinolones with two ionizable groups limit the application of gas chromatographic (GC) methods to the analysis of this group of compounds. No GC method for the analysis of quinolones has been reported. However, the fact that the quinolone antibiotics possess two nitrogen atoms and one or more fluorine atoms suggests that detection sensitivity would be high with a nitrogen/phosphorous detector (NPD) or with an electron capture detector (ECD) if a GC method was developed after removing the amphoteric properties of quinolones. Amphoteric compounds are polar and possess low volatility; therefore it is necessary to carry out derivatization to remove lipophobic propertities and make the compounds more

**Table 3.1 Published HPLC methods for analysis of pefloxacin and its metabolites**

| Reference | Sample preparation | Mobile phase | Column | Detection | Sensitivity (ng/ml) |
|---|---|---|---|---|---|
| Metz *et al.* (1990) | Hepatocyte:<br>protein pricipitated by acetonitrile/perchloric acid (4/1), supernatant was injected. | NA[1] | NA | F[2] | NOR[3] (M1): 1<br>PNO[4] (M2): 0.5 |
| Kaye *et al.* (1986) | Plasma:<br>diluted with methanol, supernatant was injected | 20% (v/v) ACN[5] in buffer (pH 3.0);<br>Buffer components: sodium acetate, citric acid, TBAB[6], formic acid. | 250 x 4 mm LiChroCa-rt Select B C18 (7µm) | F | PEF and M1: 50 |

---

[1] NA: information not available;

[2] F: fluorescence detector;

[3] NOR: norfloxacin, metabolite of N-demethylation of pefloxacin (PEF);

[4] PNO: pefloxacin N-oxide;

[5] ACN: acetonitrile;

[6] TBAB: tert-n-butyl ammonium bromide.

## Table 3.1 *(Continued)*

| | | | | | |
|---|---|---|---|---|---|
| Montay & Tassel (1985b) | Plasma:<br>diluted with sodium phophate buffer pH 7.0, extracted with chloroform/isopentanol (9/1), dry extract in 1% ammonia was injected.<br><br>Tissue:<br>homogenized with sodium phosphate buffer (pH 7.0), the supernatant was extracted with chloroform/isopentanol (9/1), dry extract in 1% ammonia was injected. | 17.7% (v/v) ACN in buffer (pH 4.8); buffer components: sodium acetate, citric acid, triethylamine. | 100 x 5 mm Nucleosil C18 (10 µm) | F | PEF: 20, M1: 30 |
| Montay *et al.* (1983) | Plasma:<br>diluted with phosphate buffer (pH 7.4), extracted with $CHCl_3$, dry extract in 1% ammonia was injected.<br>Urine:<br>diluted with phophate buffer (pH 7.0), extracted with $CHCl_3$/isopentanol (10/1, v/v), dry extract in 1% ammonia was injected. | Time — ACN% — Flow rate (ml/min)<br>0 — 8 — 2.0<br>10 — 10 — 2.0 | 100 x 4.6 mm LiChrosorb RP-18 (10 µm) | UV | PEF & M2: 1000; M1: 3000. |

volatile so that they can be analyzed by GC. One possible advantage of applying GC over HPLC is the convenience of recording electron-impact mass spectra for structural characterization of metabolites if GC is coupled with mass spectrometry. For these two reasons, a GC/MS analytical procedure was developed for the analysis of pefloxacin and its metabolites, and the developed GC method was compared with an HPLC method.

## 3.2 EXPERIMENTAL

### 3.2.1 Materials

Pefloxacin, norfloxacin and several potential internal standards structurally related to these two compounds were synthesized by the author in the Medicinal Chemistry Laboratory in the Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta. The reagents used for these synthetic procedures were obtained mainly from Aldrich Chemical Company (Milwaukee, WI, USA). They included, 3-chloro-4-fluoroaniline, diethyl ethoxymethylenemalonate, ethyl bromide, ethyl iodide, propyl iodide, acetic anhydride, piperazine and N-methylpiperazine. Reagents used for derivatization of the quinolones [thionyl chloride ($SOCl_2$), 2,2,2-trifluoro-1-ethanol (TFE) and 2,2,3,3,3-pentafluoro-1-propanol (PFP)], were also obtained from Aldrich. Solvents [chloroform ($CHCl_3$), acetonitrile ($CH_3CN$) and methanol ($CH_3OH$)] were obtained from BDH Chemicals, Canada Limited (Toronto, ON, Canada). Sodium hydrogen phosphate (BDH), tetrabutylammonium iodide (Aldrich), triethylamine (Aldrich), sodium acetate (Fisher Scientific), citric acid (Fisher Scientific) and formic acid (Aldrich) were used for the preparation of buffer solutions utilized in the preparation of mobile phases in HPLC analyses. Reagents used in metabolic studies (β-glucuronidase Type H-1, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); nicotinamide adenine dinucleotide phosphate-sodium salt and magnesium chloride were purchased from Terochem Laboratories (Edmonton, AB, Canada) and Fisher Scientific Co. (Fair Lawn, NJ, USA), respectively; Gentest Corporation (Woburn, MA, USA) was the source of purified human CYP isozymes, CYP2D6, CYP3A4 and CYP1A1, and of control microsomal preparation.

### 3.2.2 Instrumentation

Melting points were determined on a Thomas-Koover capillary melting point apparatus and are uncorrected. Proton nuclear magnetic resonance ($^1$H NMR) spectra were acquired on a Bruker AM-300 FT NMR spectrometer using tetramethylsilane as internal standard. Positive (M+1) ion FAB spectra were recorded on a VG 70SQ mass spectrometer at the Department of Chemistry, University of Alberta. Elemental analyses were performed by personnel in the Chemistry Department, University of Alberta, and in the Analytical Section of Synphar Laboratories, Edmonton.

GC analyses of trifluoroethyl or pentafluoropropyl esters of quinolones were performed under three different conditions:

a) A Perkin-Elmer Sigma 3B gas chromatograph (GC) was equipped with an ECD and a DB-5 (10m x 0.1mm ID x 0.1μm film thickness) fused-silica capillary column. Helium was the carrier gas at a flow rate of 0.6 ml/sec. The injection system was operated in the splitless mode. The injector and detector were maintained at 250°C and 305°C, respectively. The oven temperature was programmed to increase from 150°C to 280°C at 16°C/min.

b) A Varian 6000B GC was equipped with an ECD and a DB-5 (13m x 0.1mm ID x 1.0μm film thickness) fused-silica capillary column. Helium was the carrier gas at a flow rate of 0.6 ml/sec. The injection system was operated in the splitless mode. The injector and detector were maintained at 250°C and 300°C, respectively. The oven temperature was programmed to increase from original temperature of 50°C to 255°C at 35°C/min, then to 270°C at 1°C/min, and finally to 280°C at 10°C/min and held for 8 min.

c) An HP 5730A GC (Hewlett Packard, Avondale, PA, USA) was equipped with an NPD. A DB-17 fused silica capillary column (15m x 0.25mm ID x 0.25μm film thickness) was used for the separation of analytes. The oven temperature was originally set at 200°C for 2 min and then increased to 280°C for 20 min at a rate of 4°C/min. Ultra-pure helium (Union Carbide, Edmonton, Canada) was used as carrier gas at a flow rate of 1 ml/min. The injector and detector temperatures were 257°C and 308°C, respectively. The chromatograms were recorded on an HP 3396A integrator (Hewlett-Packard Company, Avondale, PA, USA).

Conditions for GC analysis of the methyl ester of pefloxacin were identical to those of method c) except for temperature programming. The oven temperature was originally set at 80°C for 2 min and then increased to 280°C for 20 min at a rate of 25°C/min.

For confirmation of structures of metabolites, their mass spectra were recorded in the electron-impact mode (ionization voltage 50 eV) using a VG-7070E mass spectrometer (VG Instruments, Sunnydale, CA, USA). The GC column and temperature program used for separation of analytes were the same as those used in the GC/ECD or GC/NPD experiments. Helium was the carrier gas at a flow rate of 3 ml/min.

Unless otherwise specified, HPLC analyses of quinolones were performed on a Varian 5500 high performance liquid chromatograph (HPLC) equipped with a Varian UV-200 (variable wavelength UV/VIS) detector and set the wavelength at 278 nm. Mobile phase consisted of $CH_3CN$ and aqueous buffer which contained sodium acetate (0.02 M. 0.14%), citric acid (0.01 M, 0.235%) and triethylamine (1 ml/l), adjusted to pH 4.2 with formic acid. The flow rate of mobile phase was 2.00 ml/min. The compounds analyzed were separated at 50°C on a Lichrosorb RP-18 column (100 x 4.6mm ID; particle size of 10 μm; Alltech Chromatography, Canada). The gradient solvent program was as follows:

| Time (min) | Flow rate (ml/min) | Buffer% | $CH_3CN$% |
|---|---|---|---|
| 0 | 2.00 | 90 | 10 |
| 2.00 | 2.00 | 90 | 10 |
| 6.00 | 2.00 | 86 | 14 |
| 7.50 | 2.00 | 80 | 20 |

### 3.2.3 Apparatus

Samples were mixed using an IKA-Vibrax-VXR multitube vortex mixer (Terochem, Edmonton, Canada) and centrifuged with a Clay-Adams bench top centrifuge (Becton Dickinson and Company, Parsippany, NJ, USA). A Reacti-Therm system purchased from Pierce (Rockford, IL, USA) was used for derivatization of the analyzed compounds.

Therm system purchased from Pierce (Rockford, IL, USA) was used for derivatization of the analyzed compounds.

## 3.2.4 Synthesis of the quinolones

### 3.2.4.1 Pefloxacin

***1-Ethyl-6-fluoro-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (7)***

*Step 1 Preparation of 3-chloro-4-fluoroanilinomethylenemalonate (3).* A mixture of 3-chloro-4-fluoroaniline (**1**) (43 g, 0.288 mol) and diethyl ethoxymethylenemalonate (**2**) (62 g, 0.288 mol) was refluxed in a 250 ml flask at 140 -145°C for 2 h (Figure 3.5). The reaction mixture was cooled to room temperature and the precipitate was recrystallized from hexane as colorless crystals (**3**; 83 g, 91%): m.p. 68-70°C (reported m.p. 55-57°C, Koga *et al.* 1980).

*Step 2 Preparation of ethyl 7-chloro-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylate* (**4**) Cyclization of **3** (83 g, 0.263 mol) was carried out with Dowtherm A (200 ml, a mixture of 73.5% diphenyl ether and 26.5% diphenyl) in a 500 ml flask at 250°C for 1.5 h. When the reaction finished, the reaction mixture was filtered, washed with hot benzene and ether, and dried to yield the cyclized product (**4**; 62 g, 88%): m.p. 314-315°C (reported m.p. > 300°C, Koga *et al.* 1980).

*Step 3 Preparation of ethyl 7-chloro-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylate (5).* Compound **4** (62 g, 0.230 mol) and potassium carbonate ($K_2CO_3$) (63 g, 0.456 mol) were refluxed in N,N-dimethylformamide (DMF) (200 ml) for 1 h at 110°C. Ethyl iodide (120 g, 0.770 mol) was added to this mixture dropwise, and the refluxing was continued for another 6.5 h. At the end of the reaction, the mixture was cooled to room temperature and poured into

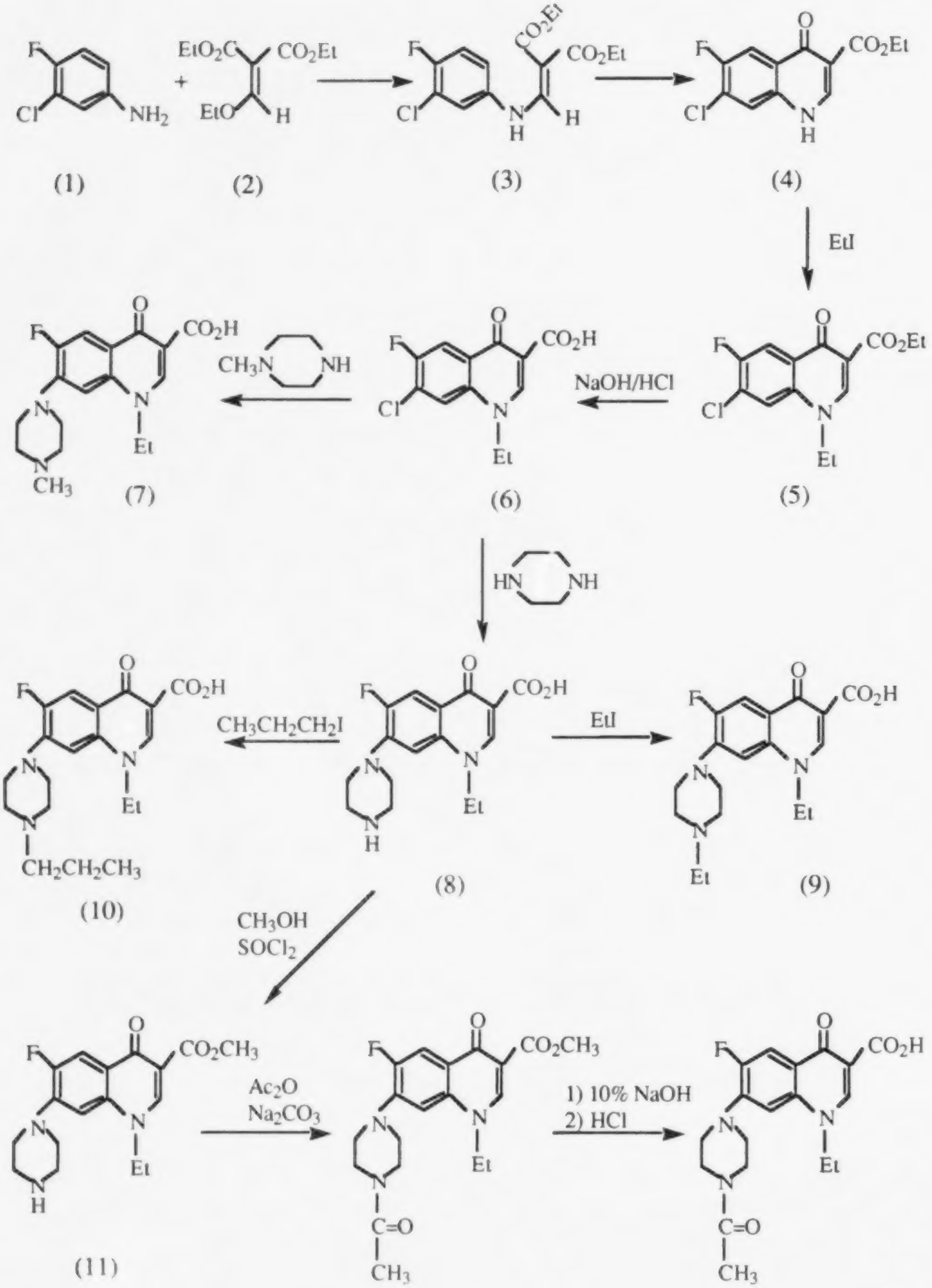

(1)
(2)
(3)
(4)
EtI
(5)
NaOH/HCl
(6)
(7)
(8)
CH₃CH₂CH₂I
EtI
(9)
(10)
CH₃OH
SOCl₂
(11)
Ac₂O
Na₂CO₃
(12)
1) 10% NaOH
2) HCl
(13)

Figure 3.5 Synthetic routes for pefloxacin (7), norfloxacin (8), ISE (9), ISP (10) and ISA (13).

a cold saturated sodium chloride (NaCl) solution. The solid was filtered, washed with water and recrystallized from ethyl acetate to give the product (**5**; 34 g, 50%): m.p. 138-140°C (reported m.p. 142-143°C, Koga *et al.* 1980).

*Step 4 Preparation of 7-chloro-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid (**6**).* A mixture of **5** (34.4 g, 0.115 mol) and 10% NaOH (300 ml) was refluxed for 1.5 h. To the soluble portion of reaction mixture was added 2 N HCl to precipitate out the product. The solid was collected and dried to give **6** (24 g, 77% ): m.p. 278-280°C (reported m.p. 284-285°C, Koga *et al.* 1980).

*Step 5 Preparation of 1-ethyl-6-fluoro-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (7).* A mixture of **6** (24 g, 0.089 mol) and 1-methylpiperazine (45 g, 0.447 mol) in a sealed tube was heated at 155-160°C for 4 h in an oil bath. After removing the solvent, the solid was recrystallized from $CH_3CN$ and dried to yield pefloxacin (**7**; 17 g, 57%): m.p. 270-272°C (reported m.p. 272-274°C, Koga *et al.* 1980). NMR and MS data were identical to the reported data (Koga *et al.* 1980).

### 3.2.4.2 Norfloxacin

***1-Ethyl-6-fluoro-1,4-dihydro-7-piperazinyl-4-oxo-3-quinolinecarboxylic acid (8)***

A mixture of 15 g (0.06 mol) of **6** and piperazine (24 g, 0.28 mol) was heated up to 155-160°C for 6 h in an oil bath. At the end of the reaction, the reaction mixture was cooled to room temperature. The solid that formed was filtered and recrystallized from $CH_3OH$ to give norfloxacin (**8**; 11 g, 55%): m.p. 220-222°C (reported m.p. 227-228°C, Koga *et al.* 1980). NMR and MS data were identical to the reported data (Koga *et al.* 1980).

### 3.2.4.3 Internal standard 1 (ISE)

***1-Ethyl-6-fluoro-7-(4-ethyl-1-piperazine)-4-oxo-3-quinoline-carboxylic acid (9)***

An $CH_3CN$ (5 ml) solution of **8** (0.5 g, 1.57 mmol) and ethyl iodide (1.3 g, 8.09 mmol) in a capped test tube (PYREX, 20mm o.d. x 150mm) was stirred in a water bath at 100°C for 5 h and was then cooled. The solid was filtered and mixed with saturated sodium bicarbonate ($NaHCO_3$) solution (20 ml). The resulting mixture was extracted with $CHCl_3$ (50 ml) twice. The combined $CHCl_3$ layer was washed ($H_2O$), dried (sodium sulphate, $Na_2SO_4$) and concentrated, giving the product (**9**; 0.27 g, 50%): m.p. 248-250°C (reported m.p. 251-253°C, Koga *et al.* 1980).

$^1H$ NMR ($CF_3COOD$): $\delta$ 1.58 (t, 3H, N-$CH_2CH_3$, $J_{H\text{-}H}$ = 8 Hz); 1.80 (t, 3H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 3.50 (q, 2H, N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 3.76-4.30 (m, 8H, 4 x N-$CH_2$); 4.90 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 7.55 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.7 Hz); 8.35 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 12.4 Hz); 9.34 (s, 1H, 2-CH).

### 3.2.4.4 Internal standard 2 (ISP)

***1-Ethyl-6-fluoro-1,4-dihydro-7-(4-propyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (10)***

An $CH_3CN$ solution of **8** (0.5 g, 1.57 mmol) and propyl iodide (1.5 g, 8.82 mmol) was placed in a capped test tube and heated at 100°C for 5 h. After cooling to room temperature, the solvent was removed. The solid was mixed with saturated $NaHCO_3$ solution (20 ml). The mixture was extracted with $CHCl_3$ (50 ml) twice. The combined $CHCl_3$ layer was washed ($H_2O$), dried ($Na_2SO_4$) and concentrated, resulting in the product (**10**; 0.32 g, 56%): m.p. 238°C.

$^1$H NMR ($CF_3COOD$) δ 1.16 (t, 3H, N-$CH_2CH_2CH_3$, $J_{H\text{-}H}$ = 7.4 Hz); 1.80 (t, 3H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 2.00 (sextet, 2H, N-$CH_2CH_2CH_3$, $J_{H\text{-}H}$ = 8 Hz); 3.37-3.76 (m, 10H, N-$CH_2CH_2CH_3$ & 4 x N-$CH_2$); 4.92 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7.3 Hz); 7.53 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.8 Hz); 8.34 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 12.4 Hz); 9.34 (s, 1H, 2-CH).

### 3.2.4.5 Internal standard 3 (ISA)

***1-Ethyl-6-fluoro-7-(4-acetyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid (13)***

To a $CH_3OH$ (5 ml) solution of **8** (0.6 g, 1.75 mmol) in a test tube cooled in an ice bath, thionyl chloride (365 μl, 5.00 mmol) was added dropwise. The test tube was capped and heated up to 100°C for 1 h in a water bath. After cooling to room temperature, the solvent was removed and the dried solid (**11**) was collected in a round-bottom flask. To this flask was added $CH_2Cl_2$ (3 ml), acetic anhydride (330μl, 3.5 mmol) and a 1 M $Na_2CO_3$ (3 ml) solution, and the mixture was stirred for 1 h at room temperature. The $CH_2Cl_2$ layer was separated and concentrated, giving compound **12**. To the compound **12,** 10% NaOH (20 ml) was added. The mixture was stirred and heated at 100°C for 30 min. After cooling, the solution was neutralized with HCl and extracted with $CHCl_3$, which was dried ($Na_2SO_4$) and concentrated to give the product (**13;** 0.32g, 50%): m.p. 292-294°C (reported m.p. 300°C, Koga *et al.* 1980).

$^1$H NMR ($CF_3COOD$) δ 1.80 (t, 3H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 2.65 (s, 3H, N-$COCH_3$); 3.91-4.21 (m, 8H, 4 x N-$CH_2$); 4.87 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 7.4 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.8 Hz); 8.31 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 12.8 Hz); 9.31 (s, 1H, 2-CH).

## 3.2.5 Derivatization of quinolones

### 3.2.5.1 Esterification with methanol

A $CH_3OH$ (5 ml) solution of pefloxacin (20 mg) and $SOCl_2$ (365 µl, 5.00 mmol) was stirred in a capped test tube at 100°C for 1 h (Figure 3.6). The solvent was removed. The solid was treated with saturated $NaHCO_3$ solution, and was extracted with $CHCl_3$. Evaporation of $CHCl_3$ yielded the derivatized product (**14**; 11.5 mg, 55%): m.p. 179°C.

MS: *m/z* 348.02 $(M+1)^+$ (FAB* ); 347 $(M^+)$ (EI** ).

$^1H$ NMR ($CF_3COOD$) δ 1.54 (t, 3H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 6.2 Hz); 2.35 (s, 3H, N-$CH_3$); 2.60–3.27 (m, 8H, 4 x N-$CH_2$); 3.84 (s, 3H, $COOCH_3$); 4.16 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 6.2 Hz); 6.69 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 7.0 Hz); 8.11 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 12.2 Hz); 8.42 (s, 1H, 2-CH).

### 3.2.5.2 Esterification with 2,2,2-trifluoroethanol (TFE)

To an $CH_3CN$ (5 ml) solution of TFE (1 ml, 14 mmol) and pefloxacin (20 mg, 0.05 mmol) in a test tube in an ice bath, $SOCl_2$ (73 µl, 1 mmol) was added dropwise. The test tube was capped and heated at 100°C for 2 h. The solvent was removed and the solid was treated with saturated $NaHCO_3$ (1 ml) and extracted with $CHCl_3$ (4 ml) twice. Concentration of the combined $CHCl_3$ layers gave the derivatized product (**15**; 12.5 mg, 60%): m.p. 160°C.

$^1H$ NMR ($CF_3COOD$) δ 1.58 (t, 3H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 2.42 (s, 3H, N-$CH_3$); 2.70–3.32 (m, 8H, 4 x N-$CH_2$); 4.24 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 4.71 (t, 2H, $CH_2CF_3$, $J_{H\text{-}F}$ = 8.6 Hz); 6.77 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 7.2 Hz);

---

[*] FAB: fast atom bombardment;

[**] EI: electron impact.

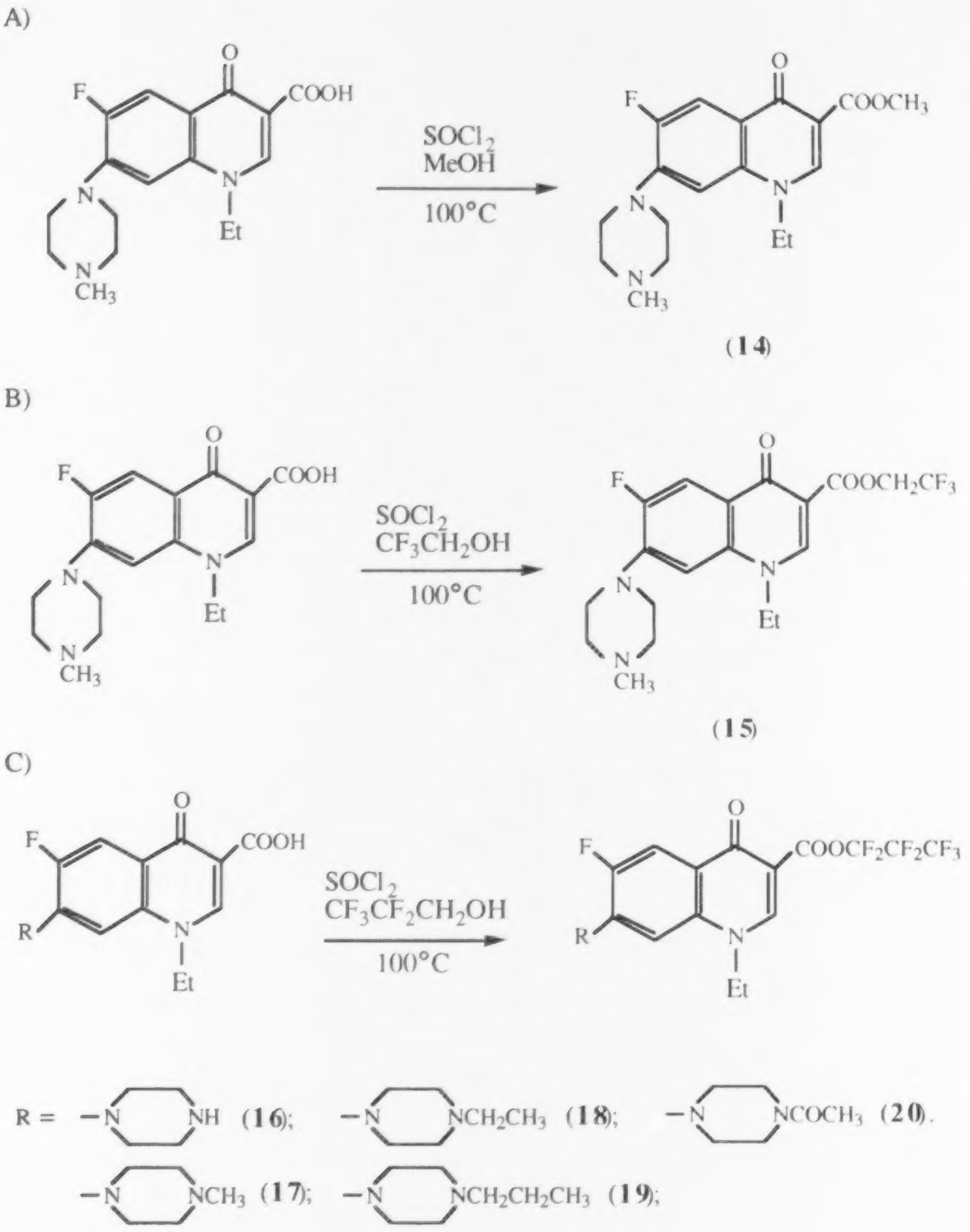

A)
F
O
COOH
N
N
CH3
N
Et
SOCl2
MeOH
100°C
COOCH3
(14)
B)
SOCl2
CF3CH2OH
100°C
COOCH2CF3
(15)
C)
R
SOCl2
CF3CF2CH2OH
100°C
COOCF2CF2CF3
R =
NH (16);
NCH3 (17);
NCH2CH3 (18);
NCH2CH2CH3 (19);
NCOCH3 (20).

**Figure 3.6 Derivatizations of quinolones.**

8.12 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 14 Hz); 9.31 (s, 1H, 2-CH).

### 3.2.5.3 Esterification with 2,2,3,3,3-pentafluoropropanol (PFP)

Derivatization of each quinolone (norfloxacin, pefloxacin, ISE, ISP and ISA) with PFP followed exactly the same procedure as described immediately above except PFP was used instead of TFE.

The melting points, NMR, MS and elemental analysis data of these products derivatized in this manner (**16**, **17**, **18**, **19** and **20**) are depicted in Table 3.2.

## 3.2.6 Analysis of quinolones

### 3.2.6.1 Sample preparation

#### *3.2.6.1.1 Preparation of standard solutions*

A standard solution of pefloxacin was prepared in distilled water at a concentration of 0.5 mg/ml. The internal standard (ISP) solution was prepared at a concentration of 0.5 mg/ml.

#### *3.2.6.1.2 Extraction*

After the addition of internal standard solution (ISP, 100 μl) and potassium phosphate buffer solution (pH 7.8; 500 μl) to 1 ml of urine sample, the mixture was shaken with $CHCl_3$ (5 ml) for 15 min then centrifuged at 1000 x g for 5 min. This extraction procedure was repeated. The combined $CHCl_3$ layer was washed with 2 ml $H_2O$, transferred to a clean tube, and evaporated to dryness under a stream of nitrogen.

For HPLC analysis, the extract obtained was dissolved in mobile phase (100 μl) (section 3.2.2), an aliquot (20 μl) was injected onto the HPLC. For GC

## Table 3.2 Characteristics of quinolones derivatized with 2,2,3,3,3-pentafluoropropanol (PFP)

| No. | Compound | M. Wt. | NMR | MS | Elemental analysis (Cal.; Found) | Melting point (°C) |
|---|---|---|---|---|---|---|
| **16** | norfloxacin-PFP<br>$C_{19}H_{19}F_6N_3O_3$ | 451.41 | δ 1.57 (t, 3H, Ar-N-$CH_2\mathit{CH_3}$, $J_{H\text{-}H}$ = 7.2 Hz); 3.12-3.24 (m, 8H,4 x NH-$\mathit{CH_2}$); 4.22 (q, 2H, Ar-N-$\mathit{CH_2}CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 4.76 (t, 2H, $\mathit{CH_2}CF_2CF_3$, $J_{H\text{-}F}$ = 13 Hz); 6.75 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.8 Hz); 8.08 (d,1H, 5-CH, $J_{H\text{-}F}$ = 13.3 Hz); 8.39 (s,1H, 2-CH). | $(M+1)^+$: 452. | C% 50.55; 49.09<br>H% 4.25; 4.44<br>N% 9.30; 8.97 | 165-167 |
| **17** | pefloxacin-PFP<br>$C_{20}H_{21}F_6N_3O_3$ | 465.43 | δ 1.56 (t, 3H, Ar-N-$CH_2\mathit{CH_3}$, $J_{H\text{-}H}$ = 7.2 Hz); 2.39 (s, 3H, N-$\mathit{CH_3}$); 2.64 - 3.29 (m, 8H, 4 x N-$\mathit{CH_2}$); 4.22 (q, 2H, Ar-N-$\mathit{CH_2}CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 4.76 (t, 2H, $\mathit{CH_2}CF_2CF_3$, $J_{H\text{-}F}$ = 13 Hz); 6.74 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.87 Hz); 8.08 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 13.3 Hz); 8.40 (s,1H, 2-CH). | $(M+1)^+$: 466. | C% 51.61; 51.61<br>H% 4.56; 4.52<br>N% 9.02; 8.78 | 173-175 |
| **18** | ISE-PFP<br>$C_{21}H_{23}F_6N_3O_3$ | 479.47 | δ 1.15 (t, 3H, Ar-N-$CH_2\mathit{CH_3}$, $J_{H\text{-}H}$ = 7.2 Hz); 1.56 (t, 3H, Ar-N-$CH_2\mathit{CH_3}$, $J_{H\text{-}H}$ = 7.2 Hz); 2.52 (q, 2H, N-$\mathit{CH_2}CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 2.68-3.30 (m, 8H, 4 x N-$CH_2$); 4.21 (q, 2H, Ar-N-$\mathit{CH_2}CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 4.75 (t, 2H, $\mathit{CH_2}CF_2CF_3$, $J_{H\text{-}H}$ = 13 Hz); 6.74 (d, 1H, 8-CH); 8.07 (d,1H, 5-CH, $J_{H\text{-}H}$ = 6.9 Hz); 8.38 (s,1H, 2-CH, $J_{H\text{-}H}$ = 13.4 Hz). | $(M+1)^+$: 480. | C% 52.60; 52.01<br>H% 4.84; 4.92<br>N% 8.76; 8.65 | 144-146 |

## Table 3.2 *(Continued)*

| | | | | | | |
|---|---|---|---|---|---|---|
| **19** | ISP-PFP<br>$C_{22}H_{25}F_6N_3O_3$ | 493.5 | δ 0.95 (t, 3H, N-$CH_2CH_2\mathit{CH}_3$, $J_{H\text{-}H}$ = 7.3 Hz); 1.56 (m, 5H, N-$CH_2\mathit{CH}_3$ & N-$CH_2\mathit{CH}_2CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 2.40 (t, 2H, N-$\mathit{CH}_2CH_2CH_3$, $J_{H\text{-}H}$ = 7.6 Hz); 2.67-3.29 (m, 8H, 4 x N-$CH_2$); 4.21 (q, 2H, N-$\mathit{CH}_2CH_3$, $J_{H\text{-}H}$ = 7.3 Hz); 4.75 (t, 2H, $CH_2CF_2CF_3$, $J_{H\text{-}F}$ = 13 Hz) 6.74 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.9 Hz); 8.09 (d,1H, 5-CH, $J_{H\text{-}F}$ = 13.3 Hz); 8.39 (s,1H, 2-CH). | $(M+1)^+$: 494. | C% 53.54; 53.33<br>H% 5.12; 5.29<br>N% 8.51; 8.44 | 154-156 |
| **20** | ISA-PFP<br>$C_{21}H_{21}F_6N_3O_4$ | 493.5 | δ 1.54 (t, 3H, Ar-N-$CH_2\mathit{CH}_3$, $J_{H\text{-}H}$ = 7.2 Hz); 2.12 (s, 3H, N-$CO\mathit{CH}_3$); 3.15-3.29 (m, 8H, 4 x N-$\mathit{CH}_2$); 4.18 (q, 2H, Ar-N-$\mathit{CH}_2CH_3$, $J_{H\text{-}H}$ = 7.2 Hz); 4.66 (t, 2H, $\mathit{CH}_2CF_2CF_3$, $J_{H\text{-}F}$ = 13 Hz); 6.69 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 7 Hz); 8.05 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 13.5 Hz); 8.36 (s,1H, 2-CH). | NA* | NA | 120-122 |

---

* NA: information not available.

analysis, the extract was analyzed after derivatization (section 3.2.6.1.3).

#### *3.2.6.1.3 Derivatization*

The tube containing this dried residue was placed in an ice bath and to it were added $CH_3CN$ (250 μl), TFE (or PFP) (250 μl) and $SOCl_2$ (19 μl). The mixture was briefly vortexed and then heated at 100°C for 1 h. After cooling, the solvent was evaporated under a stream of nitrogen. The reaction residue was mixed with saturated $NaHCO_3$ (500 μl) and extracted with $CH_2Cl_2$ (5 ml) twice. Evaporation of the organic solvent gave the pure derivatives which were reconstituted in ethyl acetate (150 μl) and ready for GC/ECD analysis.

For derivatization (esterification) with methanol in the presence of $SOCl_2$, $CH_3OH$ (500 μl) and $SOCl_2$ (50 μl) were added to the dry extract contained in a test tube which was placed in an ice bath. The reaction mixture was mixed briefly by vortex and was heated at 100°C for 1 h. After derivatization, the solvent was evaporated under nitrogen. The dry residue was treated with saturated $NaHCO_3$ solution (500 μl) and extracted with $CH_2Cl_2$ (5 ml) twice. After evaporation of the organic solvent, the dry residue was reconstituted with ethyl acetate (150 μl) and the solution (1 μl) was injected onto the GC/NPD.

#### *3.2.6.1.4 Preparation of calibration curves*

For GC analysis, the pefloxacin standard solution (section 3.2.6.1.1.1) was further diluted with drug-free urine to give concentration of 0, 2.5, 5, 10, 25, 50 and 75 μg/ml. The internal standard solution (ISP, section 3.2.6.1.1.1) (100 μl, 50 μg) was added to each one of the seven solutions. Calibration curves were constructed as the peak height ratio of pentafluoropropylpefloxacin derivative (PEF-PFP) to penta-fluoropropylated internal standard (ISP-PFP) *vs* the concentration of pefloxacin (μg/ml). For HPLC analysis, the pefloxacin standard solution was diluted with drug-free urine to give 0, 10, 25, 50, 75 and 100 μg/ml. ISP solution (100 μl, 50 μg) was added to each one of the six solutions. Calibration curves were constructed as the peak height ratio of pefloxacin (PEF) to internal standard (ISP) *vs* the concentration of pefloxacin (μg/ml). The concentration range of pefloxacin covered the concentrations found in urine samples [collected 24 h after intraperitoneal (*i.p.*) administration].

### 3.2.7 Metabolism studies

#### 3.2.7.1 Metabolism *in vivo* in the rat

Male Sprague-Dawley rats (180-200 g) were administered pefloxacin (10 mg/kg, *i.p.*) in sterile isotonic saline solution. The rats were housed individually in metabolism cages (Nalgene®, model E1000, Maryland Plastic Inc., New York, NY, USA). Free access to both water and food (Lab Chow obtained from Wayne Rodent Blox, Tekland/Premier Laboratory Diets, Bartonville, IL, USA) was allowed. A 12h day/12h night cycle was maintained throughout the duration of the experiment. Urine was collected for 24 h, then frozen at −24°C until analyzed.

Enzyme hydrolysis of the 24 h urine sample was carried out as follows. Aliquots (2 ml) of urine sample collected from drug-treated rats were adjusted to pH 5 by mixing thoroughly with sodium acetate buffer (4 ml, 0.1 M, pH 5). To the mixture, β-glucuronidase Type H-1 enzyme (750 units per ml urine) in 0.2% NaCl solution (3 ml) was added. After mixing thoroughly, the mixture was incubated at 37°C for 20 h. At the end of hydrolysis, ISP (100 μl, 50 μg) was added. The hydrolyzed urine sample was extracted, derivatized and analyzed in the same way as for the non-hydrolyzed urine sample described in sections 3.2.6.1.2 and 3.2.6.1.3.

Alkaline hydrolysis was carried out by heating the urine sample (2 ml) in NaOH (2.5 N, 2 ml) at 80°C for 1 h. ISP (100 µl, 50 µg) was added at the end of hydrolysis. The hydrolyzed sample was neutralized with 6 N HCl to pH 7, and was extracted, derivatized and analyzed in the same way as for the non-hydrolyzed urine sample described in sections 3.2.6.1.2 and 3.2.6.1.3.

### 3.2.7.2 Metabolism *in vitro* by purified CYP2D6, CYP3A4 and CYP1A1

Purified isozyme (CYP2D6 or CYP3A4 or CYP1A1) (1 mg) was incubated with pefloxacin (100 µM) in a potassium phosphate buffer (0.1 M, pH 7.4) in a final incubation volume of 1 ml . The isozyme suspension and substrate were preincubated at 37°C for 5 min. The reaction was started by the addition of NADPH-generating system (220 µl) to give final concentrations in the incubation mixture of NADPNa (1.3 mM); glucose-6-phosphate (1.3 mM); glucose-6-phosphate dehydrogenase (1 unit); and $MgCl_2$ (3 mM). The incubation was carried out in a test tube at 37°C in a shaking water bath in air for 1 h and the reaction was stopped by cooling the test tube rapidly in the ice bath. The cooled solution was extracted with $CHCl_3$ (5 ml) and the extract was processed exactly as described in sections 3.2.6.1.2 and 3.2.6.1.3. The extract was analyzed both by GC and HPLC.

To prepare a blank incubation, pefloxacin (100 µM) was also incubated with control microsomes (1 mg) that derived from a human AHH-1 TK+/– cell line which contained a low level of native human cytochrome P450 activity and was not transfected with cDNA of the appropriate CYP isozyme.

# 3.3 RESULTS

## 3.3.1 GC analysis

### 3.3.1.1 Assay

A gas chromatographic procedure for the quantification of pefloxacin and its metabolite norfloxacin and internal standard (ISP) was developed. The procedure required the esterification of the extract of the urine sample to reduce the polarity of the acid function of the quinolones. Pefloxacin, norfloxacin and ISP are well separated on a GC column and are easily detected by ECD as their pentafluoropropyl esters (Figure 3.7). The identities of the derivatives were confirmed by mass spectral analysis, and by comparing GC retention times with those of pure derivatives.

The calibration curves generated by PEF-PFP/ISP-PFP peak height ratio *vs* PEF urinary concentration showed excellent linearity ($r^2 > 0.995$) over the concentration range tested. A chromatogram of the derivatized extract from rat urine samples is depicted in Figure 3.7. Calibration curves generated on different days were reproducible. The typical calibration curve gave the following equations: $y=-0.0236+0.111x$, $r^2=0.999$ for pefloxacin. The mean slope was 0.113 with a coefficient of variation (CV) of 4.3%. The minimal pefloxacin concentration that could be quantified was 1 μg/ml. These results demonstrate that the developed GC analytical procedure was reproducible and sensitive.

### 3.3.1.2 Metabolic study in the rat

Urine of rats contained unchanged pefloxacin and two metabolites, norfloxacin and pefloxacin conjugates (unidentified). A typical GC chromatogram of the extract of rat urine collected 24 h after a single dose of 10 mg/kg pefloxacin is shown in Figure 3.7 and its GC-MS identification data are

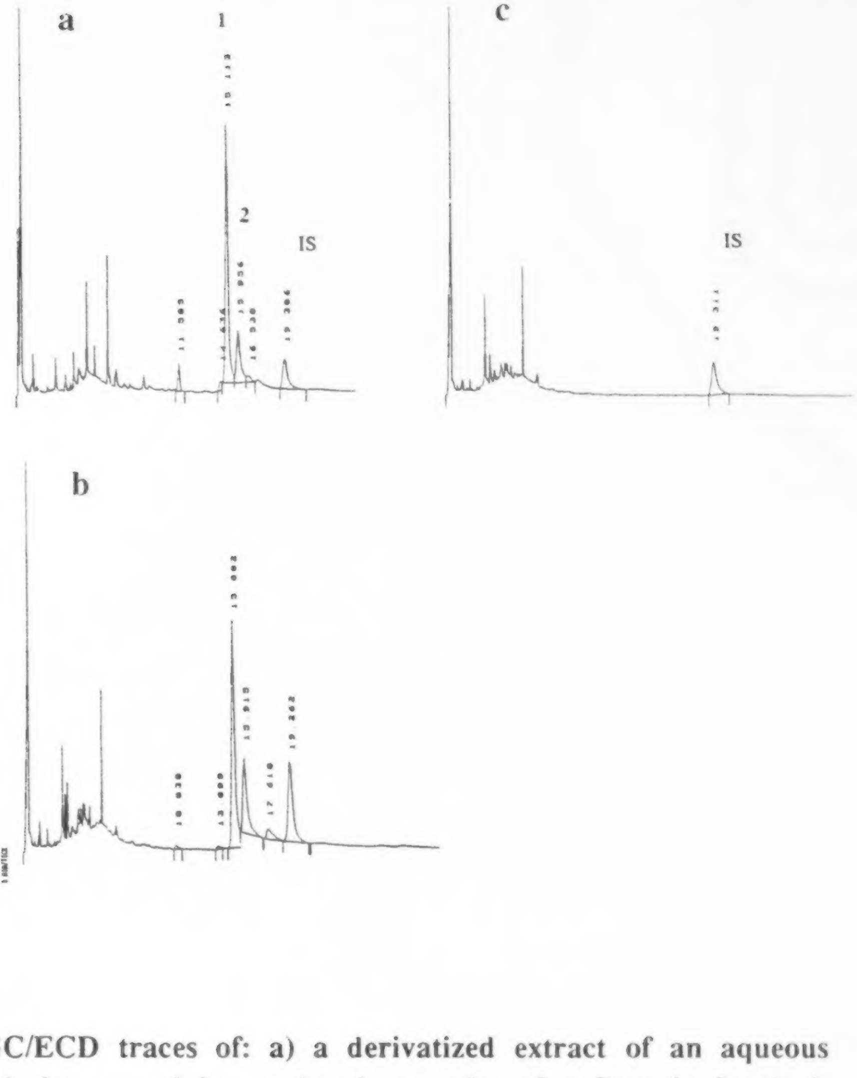


Figure 3.7 GC/ECD traces of: a) a derivatized extract of an aqueous solution containing authentic samples of pefloxacin [peak 1, $t_R$ = 15.11 min, as pefloxacin-PFP (17)], norfloxacin [peak 2, $t_R$ = 15.96 min, as norfloxacin-PFP (16)] and internal standard [IS, $t_R$ = 19.31 min, as ISP-PFP (19)]; b) a derivatized extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and c) a derivatized extract of a drug-free rat urine sample.

illustrated in Figure 3.8. The formation of unidentified pefloxacin conjugates was deduced from the higher yield of free pefloxacin obtained after alkaline hydrolysis (Figure 3.9). No pefloxacin glucuronide conjugate was formed since no difference between the total and free levels of pefloxacin was observed after β-glucuronidase hydrolysis (Table 3.3). The urinary recoveries of pefloxacin before and after alkaline hydrolysis are shown in Table 3.3 and the difference was taken as the yield of unidentified pefloxacin conjugates. The recovery of the conjugate was two to three times greater than that of free pefloxacin.

#### 3.3.1.3 Metabolic study in purified isozymes (CYP2D6, CYP3A4 or CYP1A1) system

Gas chromatograms of extracted and derivatized incubation mixtures of pefloxacin with purified human CYP2D6, CYP3A4 or CYP1A1 isozyme or control microsomes are illustrated in Figure 3.10 and 3.11. A comparison of GC retention times of the eluted peaks with that of an authentic sample revealed that no norfloxacin was formed in these purified isozyme preparations.

### 3.3.2 HPLC analysis

#### 3.3.2.1 Assay

The HPLC separation of pefloxacin, norfloxacin and ISP is shown in Figure 3.12. Calibration curves plotted as the peak height ratio of pefloxacin to ISP *vs* the urinary concentration of pefloxacin were constructed and linear relationships were obtained. The typical equation for the pefloxacin calibration curve was $y=-0.0195+0.0349x$, $r^2=0.999$. The mean value of the slope was 0.0342 with a CV of 13.3%. The concentration ranged studied (10-100 μg/ml) covered the expected concentrations of pefloxacin in the urine sample collected

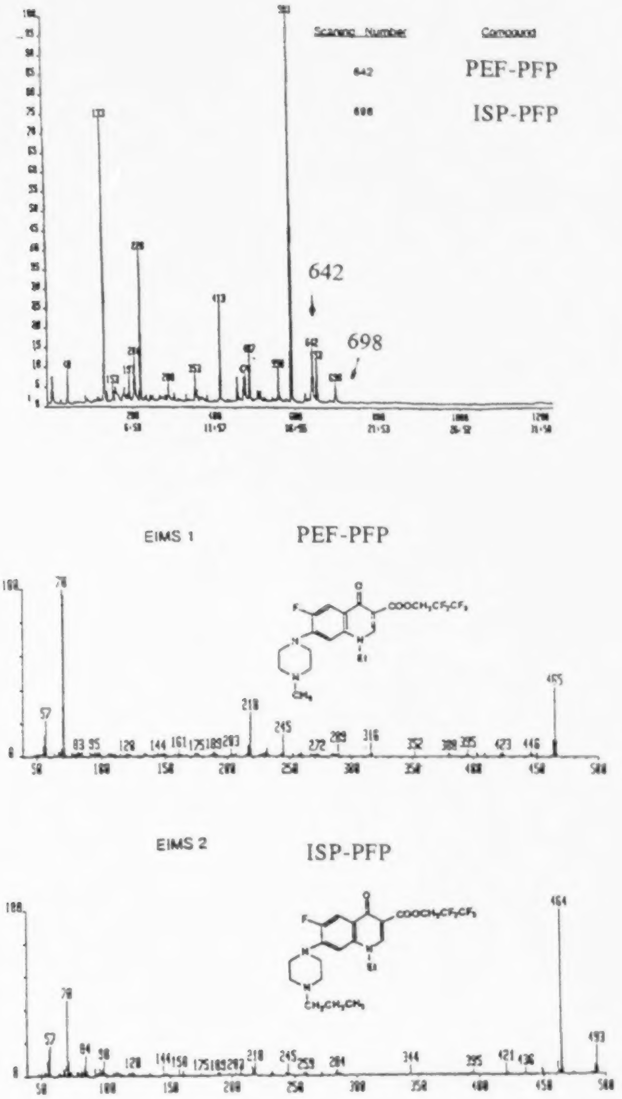


Figure 3.8 a) GC/MS trace of a derivatized extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin (peak 642, $t_R$ = 17.57 min, peak 698, $t_R$ = 19.21 min); and b) Mass spectra of pefloxacin-PFP (peak 642) and ISP-PFP (peak 698).

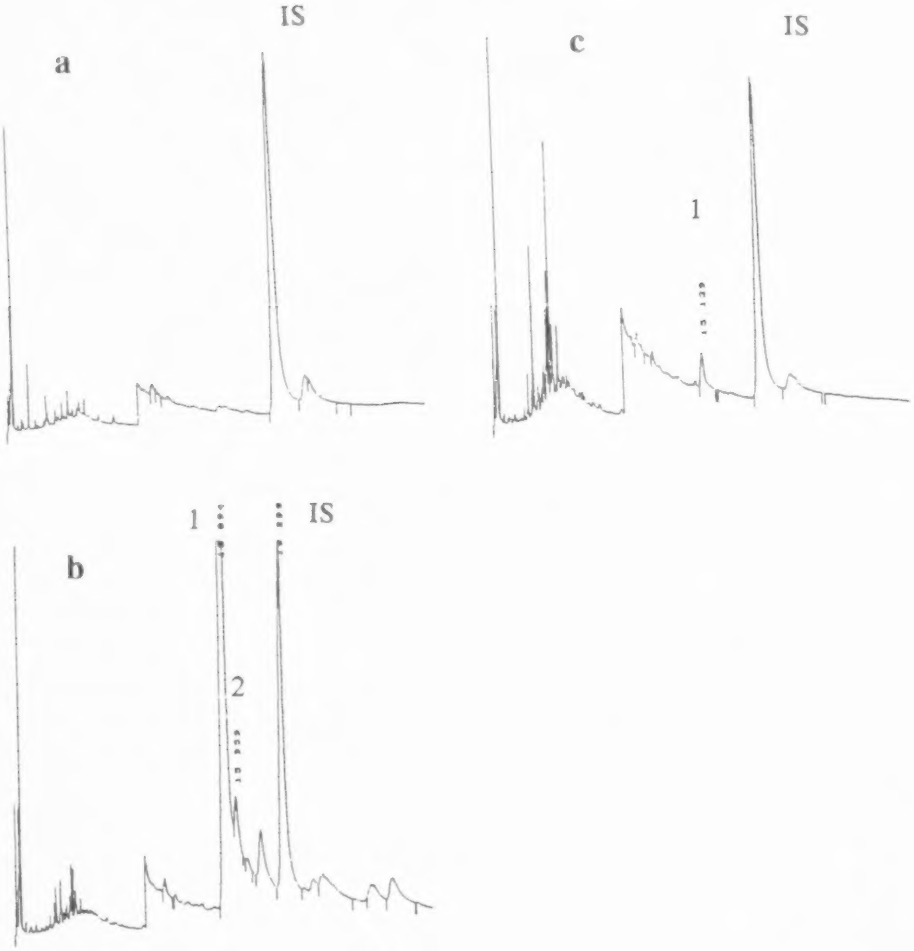


Figure 3.9 GC/ECD traces of: a) a derivatized extract of an enzymatically hydrolyzed drug-free rat urine sample; b) a derivatized extract of an enzymatically hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and c) a derivatized extract of an enzymatically hydrolyzed rat urine sample collected from 24-48 h after an *i.p.* dose of pefloxacin. Peak identification: peak 1, $t_R$ = 15.09 min, as pefloxacin-PFP (17), peak 2, $t_R$ = 15.96 min, as norfloxacin-PFP (16), IS, $t_R$ = 19.30 min, as ISP-PFP (19).

## Table 3.3 Urinary recovery of pefloxacin in the rat

| Method | Mean recovery (% of dose) ± S.D. (n=5) | | |
|---|---|---|---|
| | Urine sample (24h) | Alkaline hydrolysis of urine sample (24h) | Enzymatic hydrolysis of urine sample (24h) |
| G C | 4.4 ± 0.2 | 14.1 ± 1.0 | 3.4 ± 0.6 |
| HPLC | 3.7 ± 0.4 | 16.0 ± 2.3 | 3.7 ± 0.7 |
| Montay *et al.* (1984) | 6.6 ± 0.8 | 21.3 ± 1.7 | NA[1] |

---

[1] NA: information not available.

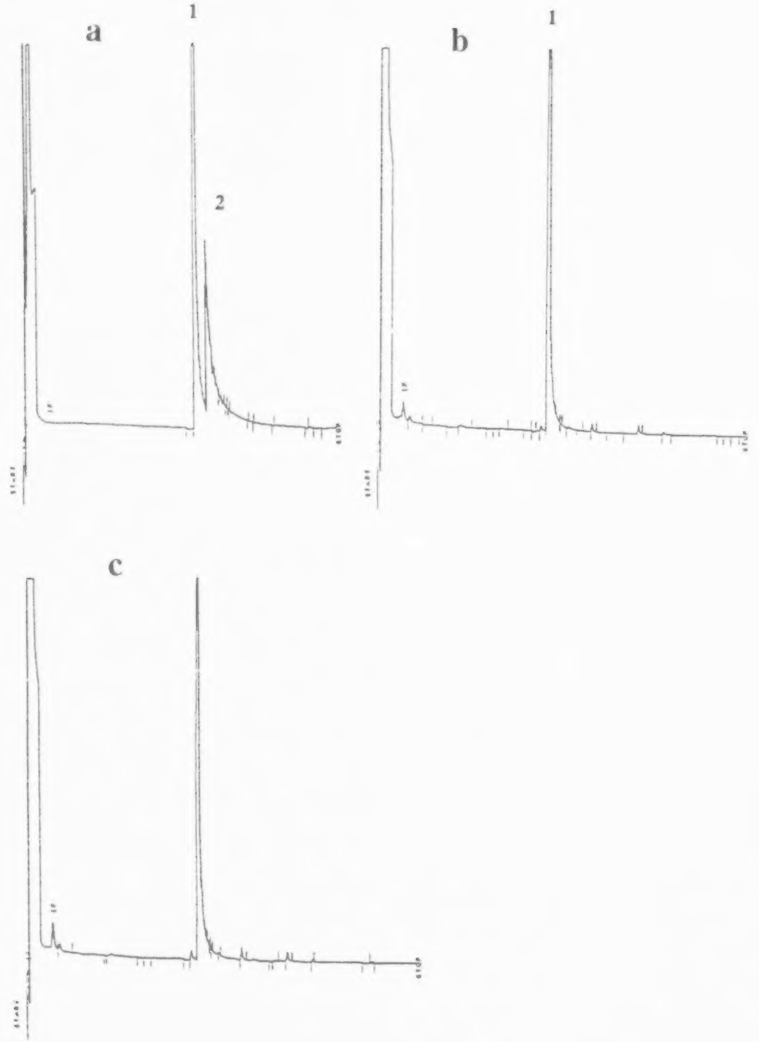


Figure 3.10 GC/NPD traces of: a) authentic sample of pefloxacin-PFP (17) (peak 1, $t_R$ = 10.33 min) and norfloxacin-PFP (16) (peak 2, $t_R$ = 10.97 min); b) a derivatized extract of a pefloxacin solution incubated with CYP3A4; and c) a derivatized extract of a pefloxacin solution incubated with CYP2D6.

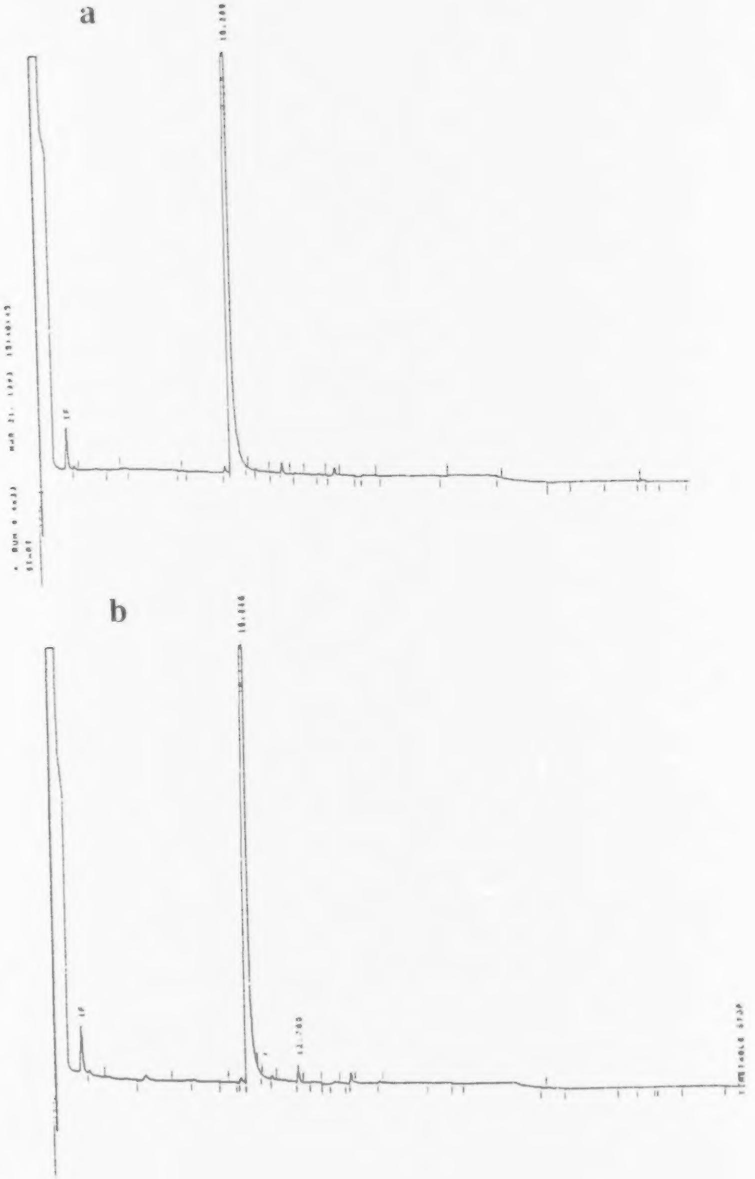


Figure 3.11 GC/NPD traces of: a) a derivatized extract of a pefloxacin solution incubated with control microsomes (peak 1, $t_R$ = 10.29 min); and b) a derivatized extract of a pefloxacin solution incubated with CYP1A1.

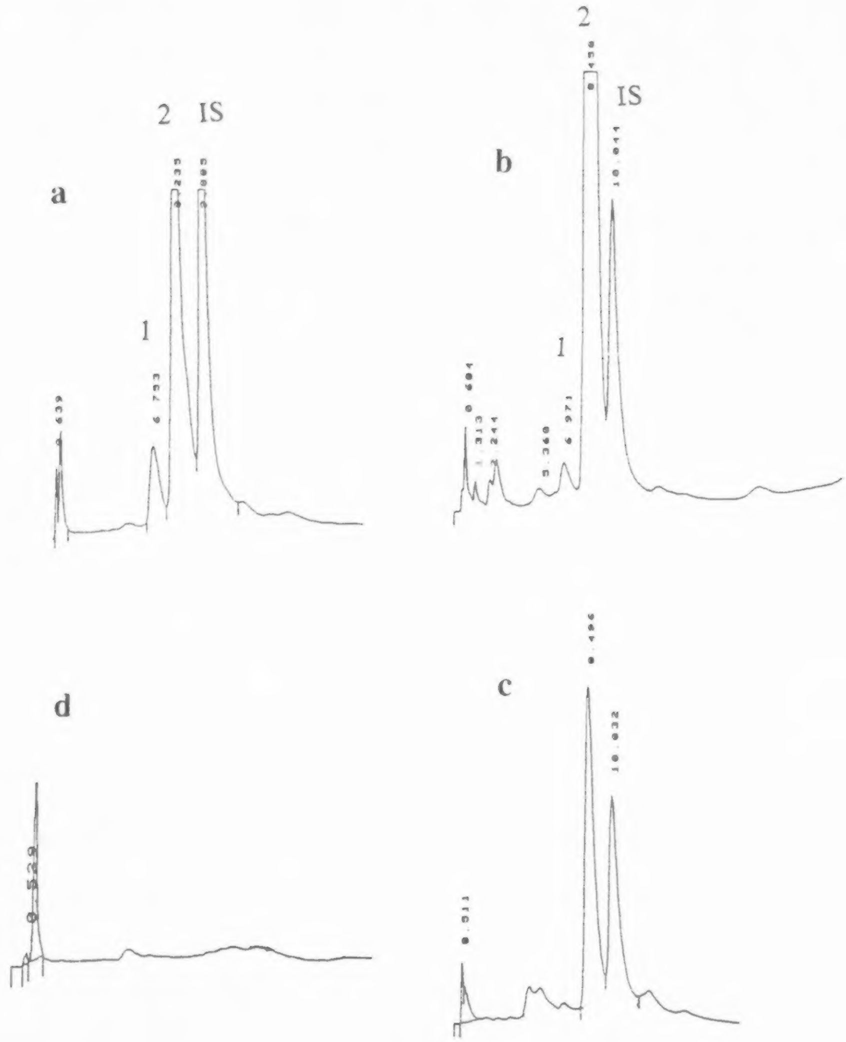


Figure 3.12 HPLC traces of: a) an extract of a rat urine sample collected for 24h after an *i.p.* dose of pefloxacin [peak 1, $t_R$ = 6.75 min, norfloxacin. peak 2, $t_R$ = 8.24 min, pefloxacin. IS, $t_R$ = 9.89 min, internal standard (ISP)]; b) an extract of an alkaline hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; c) an extract of an enzymatically hydrolyzed rat urine sample collected for 24h after an *i.p.* dose of pefloxacin; and d) an extract of a drug-free urine sample.

for 24 h after a single administration of the drug to rats.

### 3.3.2.2 Metabolic study in the rat

A typical HPLC chromatogram of an extract of rat urine is shown in Figure 3.12. Intact pefloxacin, norfloxacin and unidentified pefloxacin conjugate were the major components recovered from urine collected 24 h after a single dose of 10 mg/kg was administered to rats. The recoveries of free pefloxacin was determined as 3.7% of the dose by HPLC method (Table 3.3). The total pefloxacin recovered was measured as 16%, thus the conjugated pefloxacin was evaluated as 12.3%.

### 3.3.2.3 Metabolic study with purified isozymes (CYP2D6, CYP3A4 or CYP1A1) system

HPLC chromatograms of an incubation of pefloxacin with purified human CYP2D6, CYP3A4, CYP1A1 or control microsome are illustrated in Figure 3.13.. The HPLC retention time of an authentic sample of norfloxacin was determined. No peak of the same retention time was observed in the chromatogram of the metabolism mixtures, indicating that no norfloxacin was formed in the presence of these purified isozyme preparations.

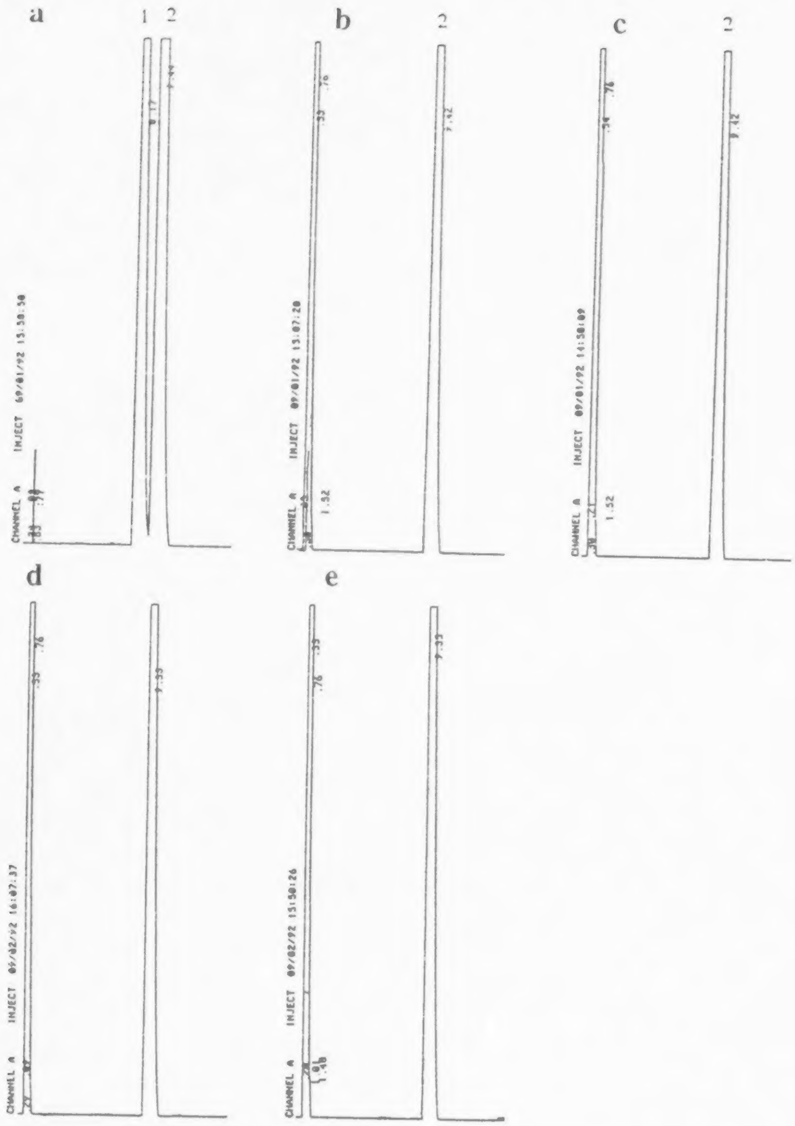


Figure 3.13 HPLC traces of: a) an authentic sample of norfloxacin (peak 1, $t_R$ = 8.17 min) and pefloxacin (peak 2, $t_R$ = 9.44 min); b) an extract of a pefloxacin solution incubated with control microsomes; c) an extract of a pefloxacin solution incubated with CYP2D6; d) an extract of a pefloxacin solution incubated with CYP3A4; and e) an extract of a pefloxacin solution incubated with CYP1A1.

## 3.4 DISCUSSION

### 3.4.1 Derivatization technique

In this study the derivatization procedures investigated included esterification with alcohols in the presence of various coupling agents such as dicyclohexylcarbodiimide (DCCI) (Kisfaludy *et al.* 1970), trifluoroacetic anhydride—boron etherate (TFAA-$BF_3$) (Dziedzic *et al.* 1976), thionyl chloride (Molnár-Perl *et al.* 1987) and 2-chloro-1-methylpyridinium iodide (Saigo *et al.* 1977). Various alcohols (*e.g.* $CH_3OH$, hexafluoro-2-propanol, pentafluoro-benzyl alcohol, pentafluoropropanol, trifluoroethanol) were used. Among the investigated techniques, $SOCl_2$ treatment gave the most satisfactory result. The same technique had been used successfully for the preparation of methyl esters of amino acids (Makisumi *et al.* 1965; Molnár-Perl *et al.* 1987).

There are no literature data on the utilization of $SOCl_2$-PFP for the esterification of fluoroquinolones. There are several advantages of incorporation of the pentafluoropropyl ester group into fluoro-quinolones: a) the polarity of the quinolones is reduced and the resulting esters elute satisfactorily from a suitable GC column; b) improved detection sensitivity by ECD; and c) structures of derivatives can be easily identified if a mass spectrometer is coupled to the GC. The developed derivatization procedure is simple and rapid. Investigation of the optimal conditions for the derivatization procedure revealed that the addition of solvent (such as chloroform or acetonitrile) in the esterification mixture favored the removal of the excess reagents, which otherwise contaminated the sample and introduced strong interfering GC peaks. However, a large excess of solvent caused the esterification procedure to be incomplete and resulted in a low yield of derivatives. The most suitable amounts of reagents and solvent were determined to give high yield of derivatives and minimal interfering peaks on the GC trace

(Table 3.4). $CHCl_3$ and $CH_3CN$ were the only two solvents chosen for the derivatization since fluoroquinolones possess poor solubility in many other organic solvents. The esterification of pefloxacin and norfloxacin was successful in both $CHCl_3$ and $CH_3CN$, but, the esterification of the internal standards, ISP and ISE, proceeded only in $CH_3CN$. Thus, $CH_3CN$ was chosen as solvent for the derivatization. In general, the yield increased as the reaction time and temperature increased (Table 3.4). When the derivatization was carried out at 100°C for longer than 2 h, the reaction mixture darkened in color, and this resulted in the appearance of interfering peaks on the GC trace. The optimal condition of the esterification was therefore fixed at 100°C for 1 h.

In addition to the pefloxacin-pentafluoropropyl ester (PEF-PFP), the pefloxacin-methyl ester (PEF-Me) and pefloxacin-trifluoroethyl ester (PEF-TEF) were also prepared using $SOCl_2$-$CH_3OH$ and $SOCl_2$—trifluoroethanol, respectively. PEF-PFP was most sensitive when analyzed by GC/ECD, followed by PEF-TEF and PEF-Me, as anticipated. Pentafluoropropyl esterification was thus selected as the derivatization method for quantification of pefloxacin using the GC analysis procedure. Among the pentafluoropropylated internal standards (ISE-PFP, ISP-PFP and ISA-PFP), ISP-PFP was the one best separated from pentafluoropropylated pefloxacin and norfloxacin. Therefore, ISP-PFP was chosen as the internal standard for use in the present study.

### 3.4.2 Metabolic study in the rat

In this study, the principal metabolites of pefloxacin in rat urine were pefloxacin conjugate and norfloxacin, which were also reported as major metabolites of pefloxacin in rat urine by Montay *et al.* (1984). After a single *i.p.* dose of 10 mg/kg to rats, the recovery of pefloxacin in 24 h urine as free intact

**Table 3.4 Optimization of derivatization conditions**

a) Effect of the reagent amount on yield of derivative[1]

| No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| $CH_3CN$ (μl) | 250 | 500 | 250 | 500 | 250 | 500 |
| $CF_3CF_2CH_2OH$ (μl) | 250 | 250 | 50 | 50 | 10 | 10 |
| $SOCl_2$ (μl) | 18.6 | 18.6 | 3.6 | 3.6 | 0.7 | 0.7 |
| Interfering peaks[2] | + | +++ | – | – | – | + |
| Yield of PEF-PFP (%) | 69 | 65 | 63 | 67 | 45 | 28 |

---

[1] Reaction conditions: 25 mg pefloxacin, 100°C for 2 h, n=4.

[2] "+" shows the existence of the interfering peaks; increased number of "+" indicates higher levels of the interfering peaks presented in the reaction mixture.

**Table 3.4** *(Continued)*

b) Effect of the reaction time on the yield of derivatives[3]

| No | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Time (hour) | 0.25 | 0.5 | 1 | 2 | 4 |
| Interfering Peaks | – | + | + | ++++ | ++++++ |
| Yield of PEF-PFP (%) | 66 | 67 | 76 | 81 | 77 |

c) Effect of the reaction temperature on the yield of derivatives[4]

| No | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Temp. (°C) | 25 | 60 | 80 | 100 |
| Interfering Peaks | – | – | + | + |
| Yield of PEF-PFP (%) | 32 | 65 | 65 | 70 |

---

[3] Reaction conditions: 25 mg pefloxacin, 100 °C, 250 ml MeCN, 250 ml $CF_3CF_2CH_2OH$, 18.6 ml $SOCl_2$, n=2.

[4] Reaction conditions: 25 mg PEF, 1 h, 250 ml MeCN, 250 ml $CF_3CF_2CH_2OH$, 18.6 ml $SOCl_2$, n=2.

drug and total pefloxacin were determined by GC as 4.4% and 14.1% of the dose, respectively, and 3.7% and 16.0%, respectively, by HPLC (Table 3.3). The amount of the glucuronide conjugate was taken to be the difference between the total and free pefloxacin levels. Thus the recovery of the glucuronide was calculated as 9.7% and 12.3% by GC and HPLC, respectively. Similar results were obtained by Montay *et al.* (1984) (Table 3.3). These comparable values, measured by GC and HPLC methods, confirmed that the developed GC analytical procedure was applicable for quantifying pefloxacin and metabolites in biological samples.

In general, there are three methods of hydrolyzing glucuronides: treatment with β-glucuronidase, HCl or NaOH. For hydrolysis of ester glucuronides such as pefloxacin-glucuronide, HCl treatment is not effective (Mandel 1971). Montay *et al.* (1984) preferred the alkaline hydrolysis method over enzymatic hydrolysis since the former reaction proceeded faster. They found that the mean urinary recovery of pefloxacin glucuronide was 14.7% of the pefloxacin dose in rat when assayed by HPLC. This value is close to the result determined by GC (9.7%) and HPLC (12.3%) (Table 3.3) using NaOH hydrolysis in the current study. However, no difference between the total and free pefloxacin levels were observed (Table 3.3) when enzymatic hydrolysis was employed, indicating that no pefloxacin glucuronide conjugate was formed. The conjugate hydrolyzed by NaOH must be some other conjugate. This observation suggested that the pefloxacin glucuronide conjugate reported by Montay *et al.* (1984) might in fact be some other conjugate since they use non-specific hydrolysis method (alkaline hydrolysis) in their analytical procedure.

### 3.4.3 Metabolic study in purified CYP isozymes

It is known that CYP2D6 is involved in N-demethylations of amitriptyline and amiflamine (Mellström *et al.* 1983; Alván *et al.* 1984) and is partially involved in N-demethylation of imipramine (Coutts *et al.* 1993). CYP3A4 is known to catalyze N-demethylation of erythromycin (Kerlan *et al.* 1992). No direct information is available on the identity of CYP isozyme(s) involved in N-demethylation of pefloxacin. The preliminary results of this study suggested that none of the isozymes CYP2D6, CYP3A4 and CYP1A1 catalyzes the N-demethylation of pefloxacin to its metabolite norfloxacin. It has also been suggested that pefloxacin inhibits N-demethylation of caffeine, which is known to be catalyzed by CYP1A2 (Robson 1992; Fuhr 1993). Incubation of pefloxacin with CYP1A2 was not conducted in this study due to the lack of a source of the isozyme.

To the best of our knowledge, no GC method has been reported for the analysis of the fluoroquinolones. A GC method useful for analysis and quantification of fluoroquinolone in biological samples has been developed successfully in the present study.

## 3.5 REFERENCES

Abramowicz M (ed). Enoxacin – a new fluoroquinolone. *Med Lett Drugs Ther* (1992) **34**: 103-105.

Alván G, Grind M, Graffner C, Sjöqvist F. Relationship of N-demethylation of amiflamine and its metabolite to debrisoquine hydroxylation polymorphism. *Clin Pharmacol Ther* (1984) **36**: 515-519.

Andriole VT. Clinical overview of the newer 4-quinolone antibacterial agents. In: *The Quinolones* (Andriole VT ed.), Academic Press, London, (1988) pp. 155-181.

Auckenthaler R, Michéa-Hamzehpour M, Pechère JC. *In vitro* activity of newer quinolones against aerobic bacteria. *J Antimicrob Chemother* (1986) **17** (Suppl. B): 29-39.

Barre J, Houin G, Tillement JP. Dose-dependent pharmacokinetic study of pefloxacin, a new antibacterial agent, in humans. *J Pharm Sci* (1984) **73**: 1379-1382.

Bergogne-Bérézin E. Pefloxacin. *Int J Antimicrob Agents* (1991) **1**: 29-46.

Beun GDM, Debrus-Palmans LL, Daniels-Bosman MSM, Blijham GH. Therapy with pefloxacin in febrile neutropenic patients. *Rev Inf Dis* (1988) **10** (Suppl.1):236.

Boerema JBJ, Pauwels R, Scheepers J, Crombach W. Efficacy and safety of pefloxacin in the treatment of patients with complicated urinary tract infections. *J Antimicrob Chemother* (1986) **17** (Suppl. B): 103-109.

Coutts RT, Su P, Baker GB, Daneshtalab M. Metabolism of imipramine *in vitro* by isozyme CYP2D6 expressed in a human cell line, and observations on metabolite stability. *J Chromatogr* (1993) **615**: 265-272.

Dabbs DJW, Limb DI, Spencer RC. The new fluoroquinolones: a review. *Med Lab Sci* (1987) **44**: 59-65.

Danan G, Montay G, Cunci R, Erlinger S. Pefloxacin kinetics in cirrhosis. *Clin Pharmacol Ther* (1985) **38**: 439-442.

Davey PG. Overview of drug interactions with the quinolones. *J Antimicrob Chemother* (1988) **22** (Suppl C): 97-107.

Dellamonica P, Bernard E, Etesse H, Garraffo R. The diffusion of pefloxacin into bone and the treatment of osteomyelitis. *J Antimicrob Chemother* (1986) **17** (Suppl. B): 93-102.

Desplaces N, Gutmann L, Acar JF. Treatment of chronic osteomyelitis (C.O.) due to *Staphylococcus spp.* or gram-negative bacteria with a new quinolone: pefloxacin (PEF). *Proc 14th Int Cong Chemother, Kyoto.* (1985) June 23-28.

Dietz WH, Cook TM, Goss WA. Mechanism of action of nalidixic acid on *Escherichia coli* III: Conditions required for lethality. *J Bacteriol* (1966) **91**: 768-773.

Dziedzic SW, Bertani LM, Clarke DD, Gitlow SE. A new derivative for the gas-liquid chromatographic determination of homovanillic acid. *Anal Biochem* (1972) **47**: 592-600.

Felmingham D. 4-Quinolone antimicrobial compounds: The relationship between *in vitro* activity and pharmacokinetic charateristics. In: *Quinolones, Proceedings of an International Telesymposium* (Fernandes PB ed.), JR Prous Science Publishers, Barcelona (1989) pp. 327-336.

Fitton A. The quinolones, an overview of their pharmacology. *Clin Pharmacokinet* (1992) **22** (Suppl. 1): 1-11.

Fuhr U, Anders EN, Mahr G, Sörgel F, Staib AH. Inhibitory potency of quinolone antibacterial agents against cytochrome P450IA2 activity *in vivo* and *in vitro*. *Antimicrob Agents Chemother* (1992) **36** (5): 942-948.

Fuhr U, Strobl G, Manaut F, Anders E, Sörgel F, Lopez-de-brinas E, Chu DTW, Pernet AG, Mahr G, Sanz F, Staib AH. Quinolone antibacterial agents: relationship between structure and in vitro inhibition of the human cytochrome P450 isoform CYP1A2. *Mol Pharmacol* (1993) **43**: 191-199.

Gibson GG, Skett P. Pharmacokinetics and clinical relevance of drug metabolism. In: *Introduction to Drug Metabolism* (Gilbson GG & Skett P eds.), Chapman & Hall, London, (1986) pp. 199-236.

Gonzalez JP, Henwood JM. Pefloxacin. A review of its antibacterial activity, pharmacokinetic properties and therapeutic use. *Drugs* (1989) **37**: 628-668.

Goueffon Y, Montay G, Roquet F, Pesson M. New synthetic antimicrobial agent: 1,4-dihydro-1-ethyl-6-fluoro-7-(4-methyl-1-piperazinyl)-4-oxo-quinoline-3-carboxylic acid (1589R.B.). *C R Acad Sci Ser* (1981) **292(3)**: 37-40.

Hajji M, El Molaghri N, Benbachir M, Marhoum El Filali K, Himmich H. Prospective randomised comparative trial of pefloxacin versus cotrimoxazole in the treatment of typhoid fever in adults. *Eur J Clin Microbiol Infect Dis* (1988) **7**: 361-363.

Harder S, Staib HA, Beer C, Papenburg A, Stille W, Shah PM. 4-Quinolones inhibit biotransformation of caffeine. *Eur J Clin Pharmacol* (1988) **35**: 651-656.

Höffler D, Schäfer I, Koeppe P, Sörgel F. Pharmacokinetics of pefloxacin in normal and impaired renal function. *Arzneimittel-Forschung* (1988) **38**: 739-743.

Holden R. Probable fatal interaction between ciprofloxacin and theophylline. *Brit Med J* (1988) **297**: 1139.

Hooper DC, Wolfson JS. Fluoroquinolone antimicrobial agents. *N Engl J Med* (1991) **324**: 384-394.

Hooper DC, Wolfson JS, Ng EY, Swartz MN. Mechanisms of action and resistance to ciprofloxacin. *Am J Med* (1987) **82** (Suppl. 4A): 12-20.

Hooper DC, Wolfson JS. The fluoroquinolones: pharmacology, clinical uses, and toxicities in humans. *Antimicrob Agents Chemother* (1985) **28**: 716-721.

Jaehde U, Malter U, Sörgel F, Gottschalk B, Wiesemann H, Stephan U, Höffler U, Schunack W. Individual factors affecting the pharmacokinetics of pefloxacin. In: *Progress in Antimicrobial and Anticancer Chemotherapy Vol 1.* (Berkarda B & Kümmerle HP eds.), Landsberg/Lech: Ecomed Verlags GmbH, (1987) pp. 883-885.

Jehl F, Koechlin C, Brogard JM, Monteil H. Biliary elimination of the fluorinated quinolones and their metabolites. In: *Quinolones* (Fernandes PB ed.), JR Prous Science Publishers, Barcelona, Spain, (1989) pp. 337-349.

Jehl F, Gallion C, Debs J, Brogard JM, Monteil H, Minck R. High-performance liquid chromatographic method for determination of ciprofloxacin in biological fluids. *J Chromatogr* (1985) **339**: 347-357.

Kaye CM, Manson J, Mitchell P, Page K. An h.p.l.c.-fluorescence method for the assay of pefloxacin and norfloxacin in plasma. *Proceedings of the BPS* December 17-19, (1986), 652P-653P.

Kayser FM. The quinolones: mode of action and mechanisms of resistance. *Res Clin Forums* (1985) **7**: 17-27.

Kerlan V, Dreano Y, Bercovici-JP, Beaune PH, Floch HH, Berthou F. Nature of cytochromes P450 involved in the 2-/4-hydroxylations of estradiol in human liver microsomes. *Biochem Pharmacol* (1992) **44**: 1745-1756.

Kisfaludy L, Roberts JE, Johnson RH, Mayers GL, Kovacs J. Synthesis of N-carbobenzoxyamino acid and peptide pentafluorophenyl esters as intermediates in peptide synthesis. *J Org Chem* (1970) **35**: 3563-3565.

Koga H, Itoh A, Murayama S, Suzue S, Irikura T. Structure-activity relationships of antibacterial 6,7- and 7,8-disubstituted 1-alkyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acids. *J Med Chem* (1980) **23**: 1358-1363.

LeBel M, Vallée F, Bergeron MG. Tissue penetration of ciprofloxacin after single and multiple doses. *Antimicrob Agents Chemother* (1986) **29**: 501-505.

LeGoffic F. Les quinolones mechanism d'action. In: *Les nouvelles quinolones* (Vashon F & Regneier B eds.), Editions Arnett, Paris, (1985) pp. 15-23.

Lewin CS, Amyes SGB. Conditions required for the bactericidal activity of pefloxacin and fleroxacin against *Escherichia coli* KL16. *J Med Microbiol* (1990) **32**: 83-86.

Lewin CS, Allen RA, Amyes SGB. Potential mechanisms of resistance to the modern fluorinated 4-quinolones. *J Med Microbiol* (1990) **31**: 153-161.

Lewin CS, Amyes SGB, Smith JT. Bactericidal activity of enoxacin and lomefloxacin against *Escherichia coli* KL16. *Eur J Clin Microbiol* (1989) **8**: 731-733.

Maesen FP, Teengs JP, Baur C, Davies BI. Quinolones and raised plasma concentrations of theophylline [Letter]. *Lancet* (1984) **2**: 530.

Mahr G, Sörgel F, Vergin H, Fabian W, Muth P, Seelmann R, Metz R. Hepatic microsomes of different species: a tool for the study of metabolism of quinolones and interactions with other drugs. *Rev Infect Dis* (1989) **11** (Suppl. 5): S1137.

Makisumi S, Saroff HA. Preparation, properties and gas chromatography of the N-trifluoroacetyl ester of the amino acids. *J Gas Chromatogr* (1965) **3**: 21-27.

Mandel HG. Pathways of drug biotransformation: biochemical conjugations. In: *Fundamentals of Drug Metabolism and Drug Disposition* (La Du BN, Mandel HG & Way EL eds.), Waverly Press Inc., Baltimore, Maryland, (1971) pp. 149-186.

Martin C, Gouin F, Fourrier F, Junginger W, Prieur BL. Pefloxacin in the treament of nosocomial lower respiratory tract infections in intensive care patients. *J Antimicrob Chemother* (1988) **21**: 795-799.

Mellström B, Bertilsson L, Lou Y-C, Säwe J, Sjöqvist F. Amitriptyline metabolism: relationship to polymorphic debrisoquine hydroxylation. *Clin Pharmacol Ther* (1983) **34**: 516-520.

Metz R, Morgenroth A, Antoni M, Mahr G, Vergin H, Muth P, Sörgel F. Hepatocytes as a tool to study quinolone metabolism. In: *Proc 3rd International Symposium on New Quinolones. Abstract #123*. Vancouver, Canada, July 12-14, (1990).

Metz R, Vergin H, Fabian W, Mahr G, Muth P, Sörgel F. Metabolism and distribution of new gyrase inhibitors in the isolated perfused rat liver. *Rev Infect Dis* (1989) **11** (Suppl.5): S1134.

Molnár-Perl I, Pintér-Szakács M, Fábián-Vonsik V. Esterification of amino acids with thionyl chloride acidified butanols for their gas chromatographic analysis. *J Chromatogr* (1987) **390**: 434-438.

Montay G, Jacquot C, Bariety J, Cunci R. Pharmacokinetics of pefloxacin in renal insufficiency. *Eur J Clin Pharmacol* (1985a) **29**: 345-349.

Montay G, Tassel JP. Improved high-performance liquid chromatographic determination of pefloxacin and its metabolite norfloxacin in human plasma and tissue. *J Chromatogr* (1985b) **339**: 214-218.

Montay G, Goueffon Y, Roquet F. Absorption, distribution, metabolic fate and elimination of pefloxacin mesylate in mice, rats, dogs, monkeys and humans. *Antimicrob Agents Chemother* (1984) **25**: 463-472.

Montay G, Blain Y, Roquet F, Le Hir A. High-performance liquid chromatography of pefloxacin and its main active metabolites in biological fluids. *J Chromatogr* (1983) **272**: 359-365.

Mulder GJ, Nagelkerke JF, Tijdens RB, Wijnands WJA, van der Mark EJ. Inhibition of the oxidative metabolism of theophylline in isolated rat hepatocytes by the quinolone antibiotic enoxacin and its metabolite oxoenoxacin, but not by ofloxacin. *Biochem Pharmacol* (1988) **37**: 2565-2568.

Neu HC. The quinolones: prospects. In: *The Quinolones* (Andriole VT ed.), Academic Press, London, (1988) pp. 235-250.

Neu HC, Chin NX, Labthavikul P, Saha G. *In vitro* activity of pefloxacin compared to that of quinolones and other antimicrobial agents. *Chemioterapia*. (1984) **3**: 235-241.

Neuman M. Comparative pharmacokinetic parameters of new systemic fluoroquinolones: a review. *Chemioterapia* (1987) **6**: 105-112.

Neuman M. Clinical pharmacokinetics of the newer antibacterial 4-quinolones. *Clin Pharmacokinet* (1988) **14**: 96-121.

Okazaki O, Kurata T, Tachizawa H. Effect of new quinolones on drug-metabolizing enzyme system of rat hepatic microsomes. *Chemotherapy* (1988) 34: 149-154.

Peixoto S. Pefloxacin versus ampicillin + gentamicin in the treatment of upper gynecological tract infections. *15th Int Cong Chemother, Istanbul* (1987) July 20-25.

Piddock LJV, Wise R. Mechanisms of resistance to 4-quinolones and clinical perspectives. *J Antimicrob Chemother* (1989) **23**: 475-480.

Piddock LJV, Wijnands WJA, Wise R. Quinolone/ureidopenicillin cross-resistance. *Lancet* (1987) **2**: 907.

Prince RA. Fluoroquinolone-drug interactions: an overview. *The Antimicrobic Newsletter* (1989) **6**: 93-99.

Robson RA. The effects of quinolones on xanthine pharmacokinetics. *Am J Med* (1992) **92** (4A): 22S-25S.

Rodrigues AD, Fernandez D, Nosarzewski MA, Pierce WM, Prough RA. Inhibition of hepatic microsomal cytochrome P-450 dependent monooxygenation activity by the antioxidant 3-tert-butyl-4-hydroxyanisole. *Chem Res Toxicol* (1991) **4**: 281-289.

Saigo K, Usui M, Kikuchi K, Shimada E, Mukaiyama T. New method for the preparation of carboxylic esters. *Bull Chem Soc Jap* (1977) **50**: 1863-1866.

Sarkar M, Polk RE, Guzelian PS, Hunt C, Karnes HT. In vitro effect of fluoroquinolones on theophylline metabolism in human liver microsomes. *Antimicrob Agents Chemother* (1990) **34**: 594-599.

Segev S, Pitlick SD, Rubinstein E. Pefloxacin for gram-negative infections in compromised patients. *Proc 15th Int Cong Chemother, Istanbul.* (1987) July 20-25.

Shen LL, Kohlbrenner WE, Weigl D, Baranowski J. Mechanism of quinolone inhibition of DNA gyrase. Appearance of unique norfloxacin binding sites in enzyme-DNA complexes. *J Biol Chem* (1989) **264**: 2973-2978.

Shen LL, Pernet AG. Mechanism of inhibition of DNA gyrase by analogues of nalidixic acid: the target of the drugs is DNA. *Proc Natl Acad Sci USA* (1985) **82**: 307-311.

Smith JT, Lewin CS. Chemistry and mechanisms of action of the quinolone antibacterials. In: *The Quinolones* (Andriole VT ed.), Academic Press, London, (1988), pp. 23-82.

Smith JT, Ratcliffe NT. Effect of pH and magnesium on the *in vitro* activity of ciprofloxacin. In: *Proc 1st Int Ciprofloxacin Workshop.* (Neu HC & Wenta H eds.), Excerpta Medica, Amsterdam, (1986) pp. 12-16.

Smith JT. Awakening the slumbering potential of the 4-quinolone antibiotics. *Pharm J* (1984) **233**: 299-305.

Sörgel F. Metabolism of gyrase inhibitors *Rev Infect Dis* (1989) **11** (Suppl. 5): S1119-S1129.

Sörgel F, Mahr G, Stephan U, Koch HU, Wiesemann HG. Absolute bioavailability and pharmacokinetics of pefloxacin in healthy volunteers. *Rev Infect Dis* (1988) **10** (Suppl.1): S93.

Sörgel F, Koch HU, Malter U, Metz R, Mahr, G, Stephan U. Metabolism of pefloxacin in human. *Rev Infect Dis* (1988) **10** (Suppl. 1): S95.

Upton RA. Pharmacokinetic interactions between theophylline and other medication (Part 1). *Clin Pharmacokinet* (1991) **20**: 66-80.

Vallée F, LeBel M, Bergeron MG. Determination of ciprofloxacin in biological samples by reversed-phase high performance liquid chromatography. *Ther Drug Monit* (1986) **8**: 340-345.

Vargas FS, Fiss E, Cukier A, Maia F, Saad E Pefloxacin treatment of severe lower respiratory tract infections. *Proc 15th Int Cong Chemother, Istanbul* (1987), July 20-25.

Webberley JM, Andrews JM, Ashby JP, Mcleod A, Wise R. Pharmacokinetics and tissue penetration of orally administered pefloxacin. *Eur J Clin Microbiol* (1987) **6**: 521-524.

Wijnands WJA, Vree TB. Interaction between the fluoroquinolones and the bronchodilator theophylline. *J Antimicrob Chemother* (1988) **22** (Suppl. C): 104-109.

Wijnands WJA, Vree TB, van Herwaarden CLA. The influence of quinolone derivatives on theophylline clearance. *Br J Clin Pharmacol* (1986) **22**: 677-683.

Wolfson JS, Hooper DC. Fluoroquinolone antimicrobial agents. *Clin Microbiol Rev* (1989) **2**: 378-424.

Wolfson JS, Hooper DC. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity *in vitro*. *Antimicrob Agents Chemother* (1985) **28**: 581-586.

Ziegler DM. Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds. In: *Enzymatic Basis of Detoxification Vol 1*. (Jacoby WB ed.), New York, Academic Press (1980) pp. 201-221.

# Chapter 4. PEFLOXACIN QUATERNARY AMMONIUM SALTS

## 4.1 INTRODUCTION

Fluoroquinolones represent an important advancement in therapy of infectious diseases and have been used widely as clinically effective antibacterial agents. However, like other antimicrobial compounds, they are not free of adverse reactions. Many of them have been shown to cause possible central nervous system (CNS) adverse reactions in low incidence (0.9 to 1.6%), such as headache, dizziness, nausea, vomiting, and tremor (Stahlmann & Lode 1988). Epileptic seizures and hallucinations occur less commonly but have been observed more frequently in patients receiving both quinolones and either theophylline or nonsteroidal anti-inflamatory drugs (NSAIDs) (Raoof *et al.* 1987; Polk 1989).

On the other hand, the low water solubility of quinolones, which is lowest at pH 7 to 9, often presents a potential problem of crystalluria and problems in formulation for intravenous (*i.v.*) use (Fernandes & Chu 1987). Crystalluria occurs more frequently in animals than in humans since animals normally have more alkaline urine (Norrby 1991). Patients with alkaline urine such as those with pyelonephritis should avoid high doses of all quinolones since crystalluria has been observed in patients and volunteers receiving high doses of norfloxacin and ciprofloxacin (Thorsteinsson *et al.* 1987).

Our interest in a search for new quinolones with improved water solubility and minimal CNS side effects prompted us to design and synthesize pefloxacin analogs with a quaternary ammonium moiety in their molecular structures. The rationale for designing the new quinolones is described briefly.

### 4.1.1 CNS side effects

Quinolone penetration into the CNS is essential for the occurrence of CNS side effects. Two barriers interfere with the penetration of drugs into the CNS — the blood-cerebrospinal fluid (CSF) barrier and the blood brain barrier (BBB). Drugs enter the CSF *via* the choroid plexus or by diffusion across the capillaries into the interstitial tissue, while drugs enter the CNS from the blood across the BBB by passive diffusion. The physicochemical properties of the quinolones such as pKa values and lipid/water partition coefficient are most important in determining CNS penetration. Pefloxacin has pKa 1 and pKa 2 values of 6.3 and 7.6, respectively (Table 4.1), and is poorly ionized in plasma (pH 7.4). Therefore, a large unionized fraction is available for CNS entry. This was confirmed by Korinek *et al.* In thirty patients who were subjected to brain tumor removal, Korinek *et al.* (1988) determined the concentration of pefloxacin in brain tissue to be in a range of 3.3 to 4.5 μg/g, and demonstrated that this high concentration of pefloxacin was attained rapidly. The lipid solubility, poor ionization, and weak protein binding of pefloxacin may contribute to this rapid achievement. Another critical factor in determining the degree of a drug penetrating into brain is its lipophilicity. Ofloxacin and pefloxacin possess higher lipid solubility than ciprofloxacin and norfloxacin. It has been determined that the concentration of ofloxacin in CSF is 50-60% of the serum concentration while ciprofloxacin in CSF is 2-19% of the serum concentration (Kitzes-Cohen 1989). Ciprofloxacin and norfloxacin are expected to have lower CNS penetration rates since their pKa values are higher and their lipid solubilities are lower than those of pefloxacin and ofloxacin.

Several *in vitro* studies have revealed that quinolones inhibit the binding of γ-aminobutyric acid (GABA) to GABA receptors, resulting in CNS excitation

**Table 4.1 Some physicochemical properties of quinolones (product information)[*]**

| Compound | Molecular weight | pKa 1 | pKa 2 |
|---|---|---|---|
| Ciprofloxacin | 368 | 6.0 | 8.8 |
| Enoxacin | 320 | 6.0 | 8.5 |
| Norfloxacin | 391 | 6.4 | 8.7 |
| Ofloxacin | 361 | 5.7 | 7.9 |
| Pefloxacin[**] | 466 | 6.3 | 7.6 |

---

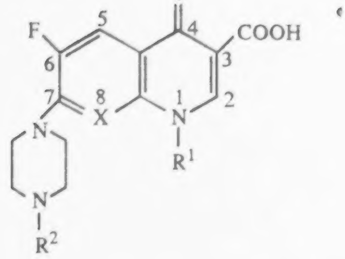
[*] Kitzes-Cohen 1989.

[**] In the form of pefloxacin dihydrate mesylate.

(Hori *et al.* 1985, 1986; Segev *et al.* 1988; Tsuji *et al.* 1988). Using mouse synaptic cell membranes, Hori *et al.* (1986) showed that norfloxacin, ciprofloxacin and enoxacin have stronger inhibitory effects than their 7-N-methylated derivatives such as pefloxacin (Figure 4.1). Thus, it is suggested that quinolones having unsubstituted piperazinyl groups might induce convulsions at lower concentrations than their substituted analogs (Fernandes & Chu 1987). This may be the reason why ciprofloxacin, norfloxacin and enoxacin show similar CNS side effects to pefloxacin *in vivo* even though they have lower CNS penetration rate. The quinolone inhibitory effect on binding of GABA to its receptors was potentiated by NSAIDs or theophylline (Hori *et al.* 1987; Segev *et al.* 1987). The 50% inhibitory dose ($ID_{50}$) of quinolones for GABA was reduced by up to 10,000-fold in the presence of NSAIDs or theophylline. In Japan, numerous cases of convulsions were reported when fenbufen, a NSAID, was coadministered with enoxacin (Polk 1989).

### 4.1.2 Structure and biological activity

The structure-activity relationships and the related synthetic chemistry of quinolones were reviewed extensively by Albrecht in 1977. Since then, comprehensive reviews on the structure-activity relationships have been provided by Mitscher *et al.* (1989), Chu and Fernandes (1989, 1991), and Rádl (1990). In the last twenty years, more than 5000 new analogs have been described in the literature. About twenty of them are either marketed or are undergoing intensive preclinical study. Because most of the clinically useful quinolones bear a fluorine atom at the C-6 position of the quinolone or naphthyridine ring system (Figure 4.1), these antibacterial agents are commonly described as fluoroquinolones.


O
4
COOH
3
2
1
N
R1
F
5
6
7
8
X
N
N
R2

| No | Compound | $R^1$ | $R^2$ | X |
|---|---|---|---|---|
| 1 | Norfloxacin | Et | H | CH |
| 2 | Ciprofloxacin | △* | H | CH |
| 3 | Enoxacin | Et | H | N |
| 4 | Pefloxacin | Et | Me | CH |

---

* cyclopropyl group

**Figure 4.1 Structures of some fluoroquinolones that show CNS side effect.**

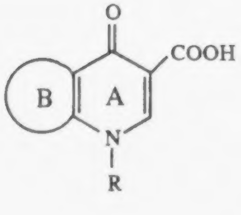


**Figure 4.2 Bicyclic heteroaromatic quinolones.**

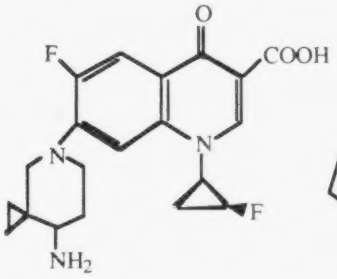
Quinolones exert their antibacterial activity through the action of penetrating bacterial cells and inhibiting DNA gyrase. Structurally, it has been shown that the bicyclic heteroaromatic system combining 1,4-dihydro-4-pyridine-3-carboxylic acid moeity A and an aromatic ring B (Figure 4.2) is necessary for binding to DNA gyrase (Chu & Fernandes 1989). Moreover, the nature of the peripheral substituents and their spatial position relative to the nucleus not only provide additional affinity for binding of the drug to the bacterial enzymes but also enhance the cell penetration of the drug and hence improve the antibacterial activity of the quinolone. The presence of a fluorine atom at the C-6 position significantly enhances the activities of both gyrase inhibition and cell penetration, leading to 1000 times higher potency than the compound in which the C-6 fluorine atom is absent (Dabbs *et al.* 1987). The nature of the C-7 substituent has a major impact on solubility which in turn controls cell permeability and pharmacokinetics (Chu & Fernandes 1991). In general, potent quinolone derivatives require lipophilic substituents at N-1 and C-8 positions for the desired pharmacokinetic profile; a basic group at C-7 position for *in vivo* activity; a fluorine atom at C-6 position for improved antibacterial potency; and a co-planar β-keto-carboxylic acid feature at C-3 and C-4 positions for hydrogen bonding with DNA nucleotides (Chu & Fernandes 1991).

***Position 1.*** Steric and regional electronic configurations of a substituent at the N-1 position have a great influence on the antibacterial activity. The optimal size of the substituent would be similar to that of ethyl or cyclopropyl groups as exemplified by norfloxacin or ciprofloxacin (Ohta & Koga 1991). Addition of one fluorine atom to the N-1 cyclopropyl group (exemplified by compound **5**) or the 1-tert-butyl substituent (exemplified by compound **6**) have been found to increase activity against gram-positive bacteria (Suto *et al.* 1992)

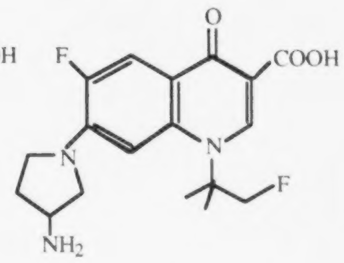
(Figure 4.3). Introduction of a fluorophenyl substituent at the N-1 position, such as in difloxacin (**8**), tosufloxacin (**9**) and temafloxacin (**10**), may also enhance the *in vitro* activity against anaerobic bacteria (Chu & Fernandes 1989) (Figure 4.3). Most recent studies suggest that the antibacterial activity of a quinolone is predominately influenced by the substituent at N-1 rather than by the group at the C-5, C-7 or C-8 position (Suto *et al.* 1992).

***Position 2***. Modifications at position 2 are rare and often lead to inactive compounds. However, bridging the N-1 substituent to C-2 may produce biologically active derivatives. In compound **7** bridging the N-1 and C-2 with a sulphur atom yielded good antibacterial activities (Chu & Fernandes 1989) (Figure 4.3). Several studies have indicated that the smaller the ring size for substituents bridging the N-1 and C-2 positions, the better the antibacterial activity (Chu & Fernandes 1991).

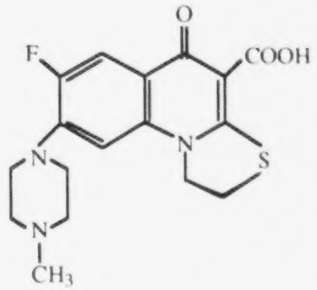
***Position 3***. A combination of the 3-carboxy and 4-oxo group is a key moiety for binding of a quinolone to DNA gyrase. Some attempts to replace the 3-carboxylic acid function with acidic groups such as sulfonic acid, acetic acid, and phosphoric acid resulted in a decrease in antibacterial activities (Albrecht 1977). A recent breakthrough is the modification at the 3-position with a bioisostere-fused isothiazolo ring as exemplified in compound **11** (Figure 4.3). These compounds exhibited higher potency and enhanced activity against DNA gyrase than their 3-carboxy analog (Chu *et al.* 1989). The isothiazolo ring system possesses an aromatic character, and the nitrogen proton is acidic and can be considered as a carboxylic acid-mimic. 3-Formyl analogs of norfloxacin, pefloxacin and ciprofloxacin have been synthesized as useful prodrugs (Kondo *et al.* 1988). The rapid *in vivo* activation of the prodrug produced much higher drug concentrations in serum compared to that yielded by the parent drug.



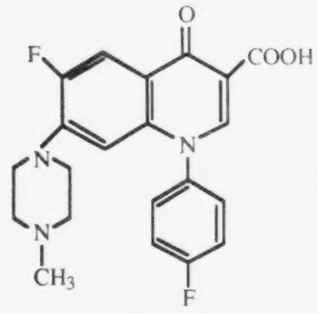
5



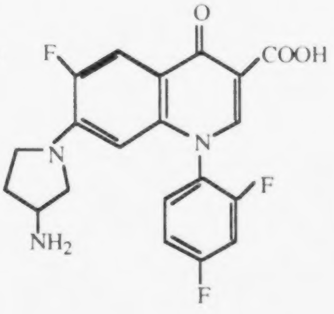
6



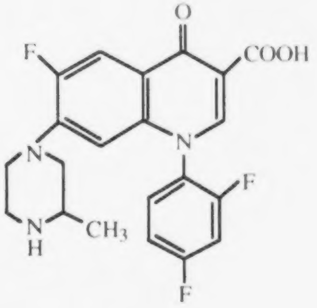
7



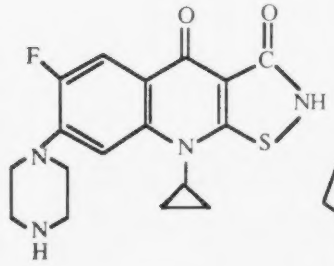
8 (Difloxacin)



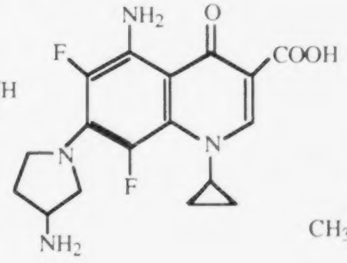
9 (Tosufloxacin)



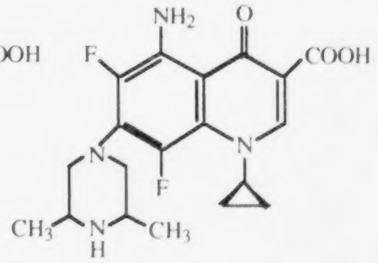
10 (Temafloxacin)



11

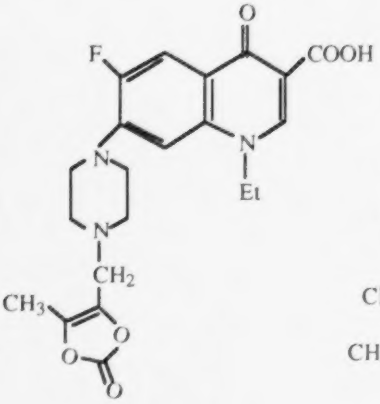


12

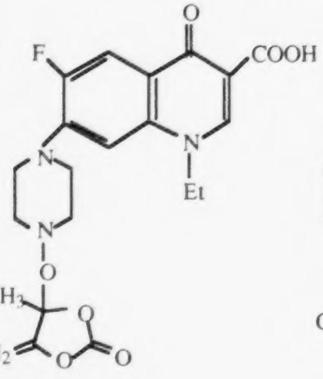


13 (Sparfloxacin)

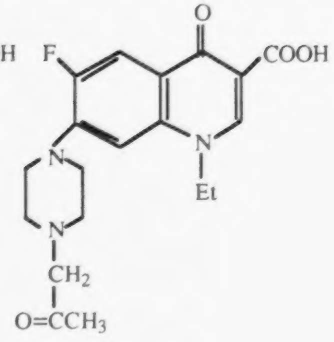
Figure 4.3 Structures of some fluoroquinolones.



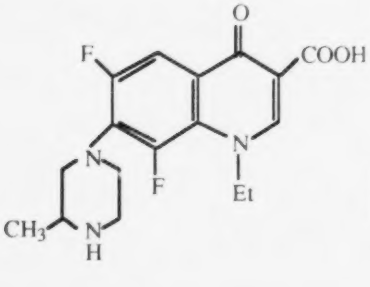
**14**



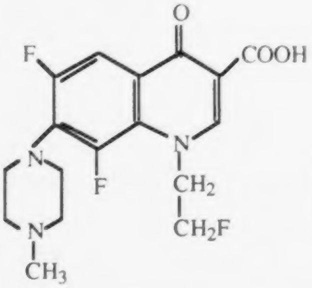
**15**



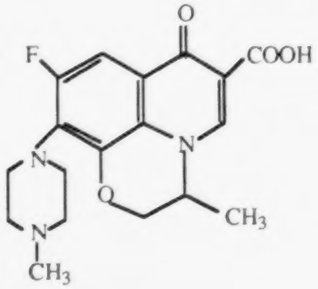
**16**



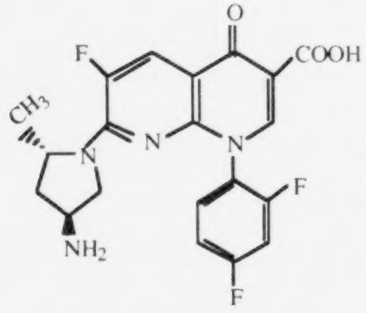
**17** (Lomefloxacin)



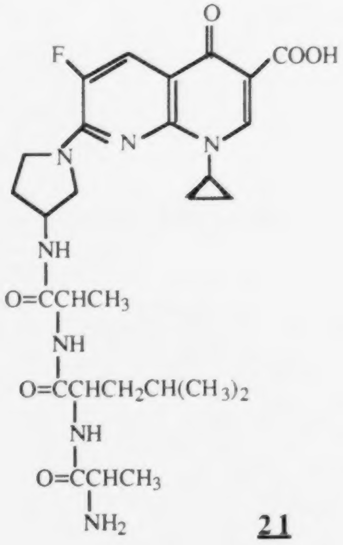
**18** (Fleroxacin)



**19** (Ofloxacin)



**20**



**21**

Figure 4.3 *(Continued)*

***Position 4***. The presence of the keto group at this position is thought to be necessary for the activity against DNA gyrase. Previous attempts to replace the 4-keto by a 4-thioxo or a 4-sulfonyl group led to inactive compounds (Albrecht 1977).

***Position 5***. Steric factors are the most important considerations in modification of the group at position 5. Of the various C-5 small substituents evaluated, such as nitro, amino, halo and alkyl groups, the addition of amino group to 6,8-difluoroquinolones was beneficial for activity. Several 6,8-difluoroquinolones having 5-amino substitution have been reported to possess enhanced *in vitro* antibacterial activity far superior to that of ciprofloxacin (Domagala *et al.* 1988). The most potent *in vivo* compound in this series is compound **12** (Figure 4.3), which is currently under evaluation as a potential drug candidate (Rádl 1990). Sparfloxacin (**13**), another 5-aminoquinolone currently under clinical development, has improved activity against gram-positive and anaerobic bacteria.

***Position 6***. Among many modifications investigated at the C-6 position (H, F, Cl, Br, $CH_3$, $SCH_3$, $COCH_3$, CN and $NO_2$), only introduction at C-6 of a fluorine atom resulted in a dramatic increase in antibacterial potency. Compared to the derivatives with no substitution at the C-6 position, the corresponding 6-fluoroquinolones showed significant improvement in both cell penetration and binding to DNA gyrase (Rádl 1990).

***Position 7***. Combination of the C-6 fluoro group with a suitable C-7 substituent is necessary for producing a quinolone with high potency and broad spectrum activity. Modification at C-7 position has been investigated extensively. The most common C-7 substituents which resulted in quinolones with enhanced antibacterial activities are (a) 1-piperazinyl group [*e.g.* norfloxacin (**1**), ciprofloxacin (**2**) and enoxacin (**3**)], (b) 4-methyl-1-piperazinyl group [*e.g.* pefloxacin (**4**), ofloxacin (**19**), difloxacin (**8**) and fleroxacin (**18**)], (c) 3-amino-1-pyrrolidinyl group [*e.g.* tosufloxacin (**9**)], (d) 3-methyl-1-piperazinyl group [found in lomefloxacin (**17**) and temafloxacin (**10**)] (Chu & Fernandes 1989) (Figure 4.3).

In general, quinolones with a 4-methylpiperazinyl group (*e.g.* pefloxacin) are more potent against gram-positive bacteria and slightly less active against gram-negative bacteria compared to their corresponding C-7 piperazinyl quinolones (*e.g.* norfloxacin). However, the quinolones with a C-7 4-methylpiperazinyl group have more favorable cell permeability which provides them excellent bioavailability. In fact, the C-7 piperazinyl derivatives normally do not have good bioavailability. Recently, several studies on the modification of the C-7 piperazinyl group of norfloxacin generated a series of prodrugs with better oral absorption (Chu & Fernandes 1991). These derivatives include compound **14**, **15** and **16** in Figure 4.3. All of them exert enhanced *in vivo* antibacterial activity over norfloxacin and result in higher plasma levels of norfloxacin after oral administration. They are thought to produce norfloxacin by oxidative metabolism *in vivo*. It is also known that insertion of a 3-aminopyrrolidinyl group at C-7 enhances the overall antibacterial spectrum of a quinolone, particularly the anti-gram-positive bacterial activity. However, quinolones with this feature have poor water solubility at pH 7.4 and may cause absorption problems in human. Water solubility can be greatly enhanced by the addition of a methyl group at the 5-position of the 3-aminopyrrolidinyl substituent as shown in compound **20**. Another example of a 7-(3-aminopyrrolidinyl) quinolone with improved water solubility is compound **21** which was formed by conjugating the parent drug with an α-amino acid or dipeptide at the 3-amino group of 3-aminopyrrolidinyl substituent. This prodrug has an increased water solubility and is rapidly hydrolysed *in vivo* to the parent compound, providing an excellent *in vivo* potency (Chu & Fernandes 1991).

It is apparent that a substituent at the C-7 position has a great influence on both antibacterial activity and biopharmaceutical features. These properties provide great flexibility in the design of the molecule, with an optimal substituent pattern to generate the ideal fluoroquinolone-incorporating features, such as greater water solubility, lower CNS side effects, improved pharmacokinetics and an improved antibacterial spectrum.

***Position 8***. Among many modifications investigated at the C-8 position, only the replacement of C-8 with a nitrogen atom (N-8) or modification of C-8 with a halogen atom (*e.g.* F or Cl) produced clinically useful quinolones. C-8-Fluoro or -chloro derivatives are generally more active *in vivo* due to better oral absorption, as exemplified by lomefloxacin (**17**) and fleroxacin (**18**) (Figure 4.3). However, their *in vitro* activities are less than those quinolones with no substitution at the C-8 position. Similarly, naphthyridine analogs in which C-8 CH moiety is replaced by a nitrogen atom are normally less active *in vitro* than their quinolone counterparts. However, this inferior *in vitro* activity is overcome by better absorption which enhances their *in vivo* activity. Ofloxacin (**19**), having a 3-methyloxazine ring between N-1 and C-8, is more potent than norfloxacin in *vitro* and has excellent *in vivo* efficacy (Norris & Mandell 1988).

### 4.1.3 Rationale for designing pefloxacin analogs

From the structure and activity relationships, it is clear that substitution at the C-7 position is an appropriate approach to structure modification to generate a quinolone with minimal CNS side effects and better water solubility. Pefloxacin was chosen as a parent drug for modification since it undergoes extensive metabolism. The 4-methylpiperazinyl group at the C-7 position of pefloxacin is not only the location where metabolism occurs but it is also a moiety that influences cell permeability. By designing an ionic substituent at C-7 position, one would expect the designed compound to have little chance to cross the BBB but would be more likely to dissolve in water. It is hypothesized at this point that once it enters the body, the designed ionic compound may be activated *in vivo* by metabolism back to pefloxacin.

Atracurium, a neuromuscular blocking drug, is an example of a quaternary ammonium salt that is degraded to form a tertiary base under physiological conditions (pH 7.4 and 37°C) (Stenlake 1983). The route of breakdown of atracurium is illustrated in Figure 4.4. Another example of a quaternary ammonium compound undergoing metabolic N-demethylation is SA-504 [1,1-dimethyl-5-methoxy-3-(dithien-2-ylmethylene)piperidinium bromide], which was metabolized *in vivo* in rat to give a tertiary amine (Meshi *et al.* 1973). The metabolic N-demethylation pathway of SA-504 is shown in Figure 4.5. Bearing in mind the idea that a quaternary ammonium compound may be metabolized *in vivo* to a pharmacologically active tertiary amine, the target compounds, PQAS-1, PQAS-2 and PQAS-3, were designed (Figure 4.6).

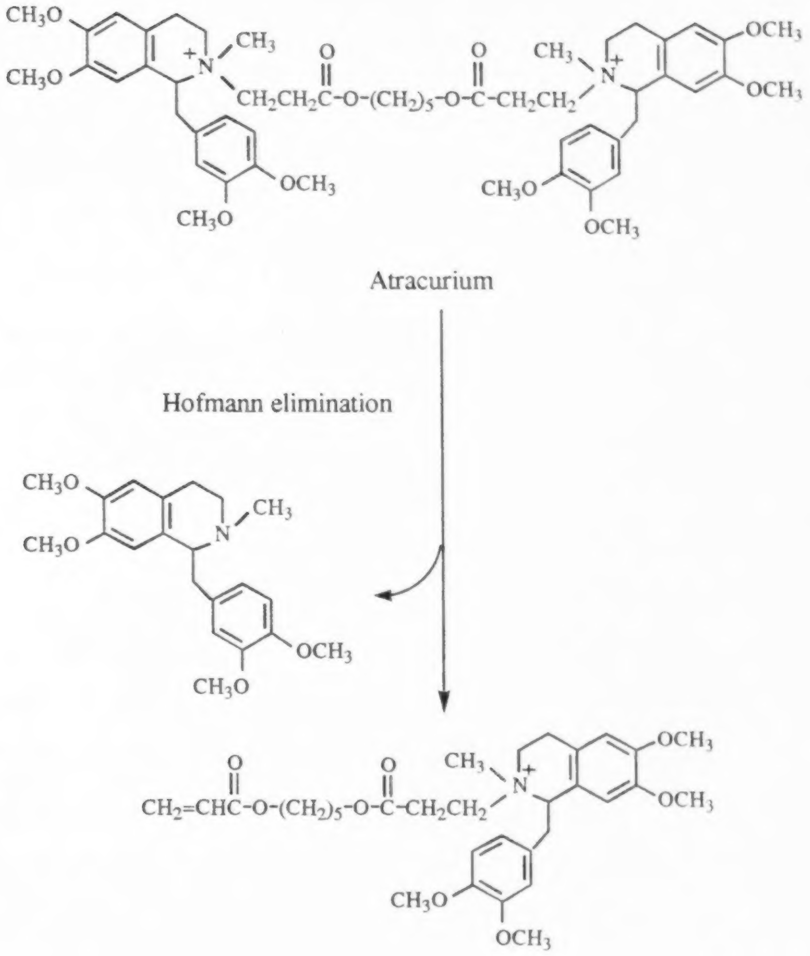
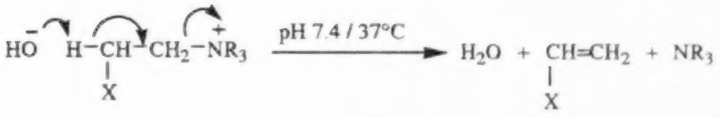

Atracurium
Hofmann elimination

**Figure 4. (a) Hofmann elimination of atracurium**



$$\overset{-}{HO}\ \ H\underset{\underset{X}{|}}{-CH}-CH_2-\overset{+}{N}R_3 \xrightarrow{pH\ 7.4\ /\ 37^{\circ}C} H_2O + \underset{\underset{X}{|}}{CH}=CH_2 + NR_3$$

**(b) Mechanism of Hofmann elimination**

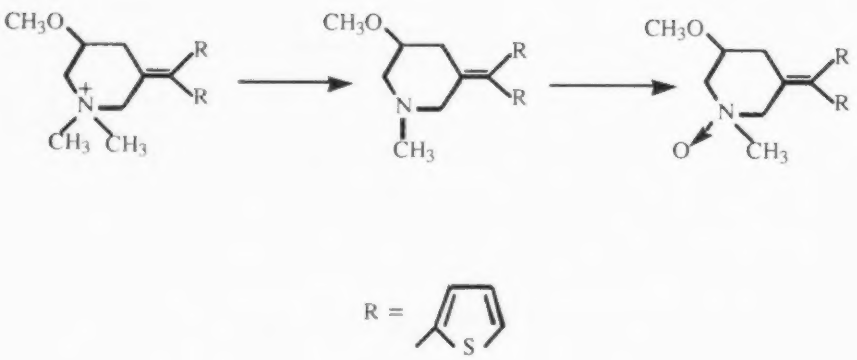

CH3O
R
R
N
CH3
CH3
CH3O
R
R
N
CH3
CH3O
R
R
N
O
CH3
R =
S

**Figure 4.5** Metabolic N-demethylation of SA-504 and N-oxidation of the primary metabolite.

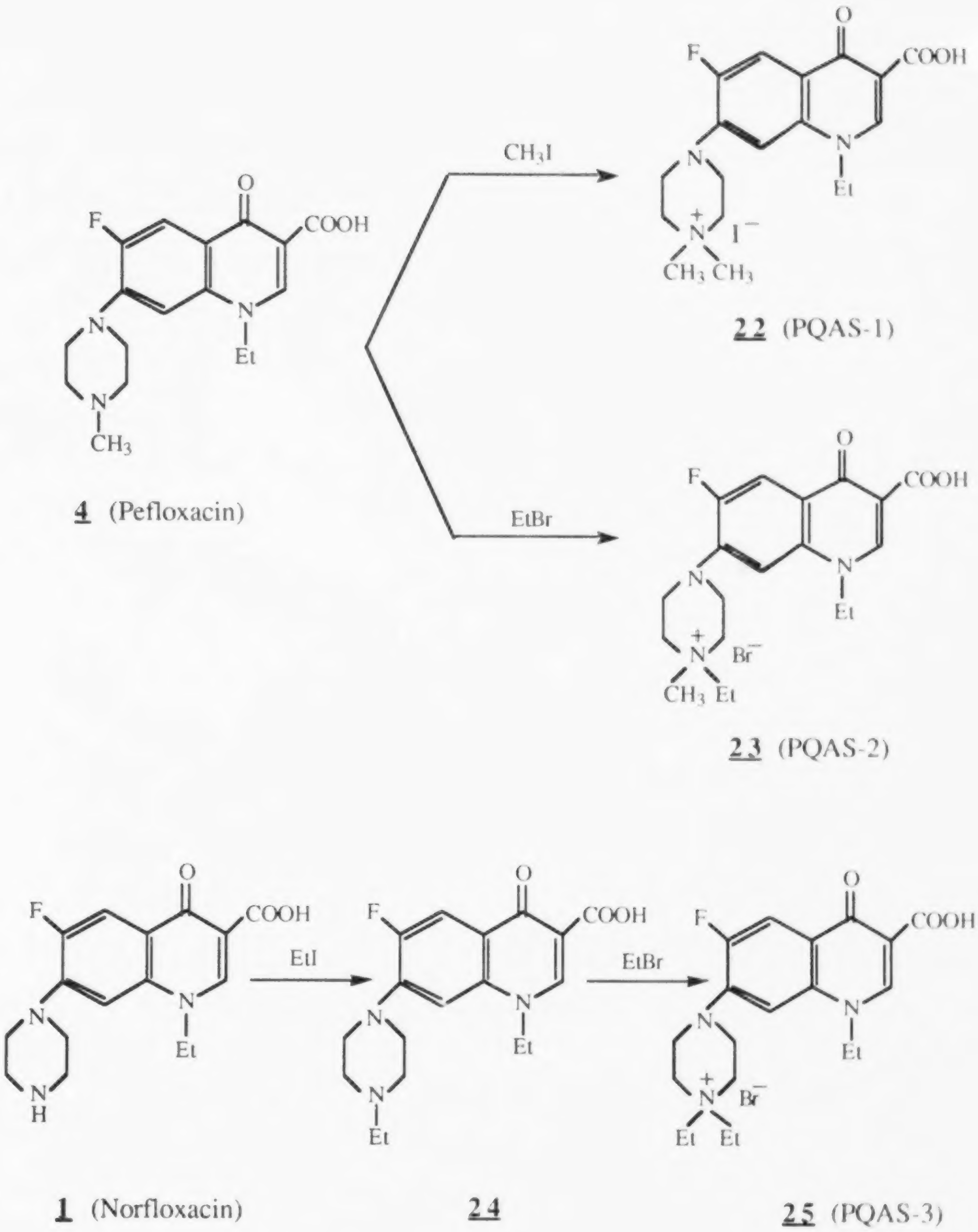

CH3I
EtBr
4 (Pefloxacin)
22 (PQAS-1)
23 (PQAS-2)
EtI
EtBr
1 (Norfloxacin)
24
25 (PQAS-3)

**Figure 4.6 The synthetic routes for fluoroquinolone quaternary ammonium salts.**

## 4.2 EXPERIMENTAL

### 4.2.1 Materials

Pefloxacin was synthesized. Methyl iodide, ethyl iodide, ethyl bromide, tetrabutyl ammonium iodide, triethylamine and formic acid were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Chloroform, acetonitrile, methanol and sodium hydrogen phosphate were obtained from BDH Chemicals, Canada Limited (Toronto, ON, Canada). Purified human CYP isozymes, CYP2D6, CYP3A4, CYP1A1 and control microsomes, were purchased from Gentest Corporation (Woburn, MA, USA).

### 4.2.2 Instrumentation

A Thomas-Koover capillary melting point apparatus was used for the determination of melting points of the sythesized compounds. Proton magnetic resonance ($^1$H NMR) spectra were recorded on a Bruker AM-300 FT NMR spectrometer. Chemical shifts were recorded in $\delta$ units relative to the internal reference, tetramethylsilane. Positive ion (M+1) FAB mass spectra were acquired on a VG 70SQ mass spectrometer at the Department of Chemistry, University of Alberta. UV spectra were recorded on a PU 8700 series, UV/Visible scanning spectrophotometer (Philips, Scientific & Analytical Equiment). UV data of all compounds were taken in saline solution.

HPLC analyses of PQAS-1 and PQAS-2 were performed on a Waters LC Module-1 instrument equipped with a Waters M-486 UV detector set at 278 nm and a Waters-715 ultra WISP autosampler. Separation was accomplished on a Lichrosorb RP-18 column with particle size of 10 µm (100 x 4.6mm ID; Alltech Chromatography, Canada). A mixture of acetonitrile in buffer which contained sodium acetate (0.02 M, 0.14%), citric acid (0.01 M, 0.235%) and triethylamine

(1 ml/l), adjusted to pH 4.2 with formic acid, was used as the mobile phase. The buffer solution and the acetonitrile were filtered to eliminate particulate matter and dissolved gases by vacuum filtration with a Nylon 66 filter (0.45-μm). The gradient solvent program was as follows:

| Time (min) | Flow (ml/min) | Buffer% | $CH_3CN$% |
|---|---|---|---|
| 0 | 2.00 | 90 | 10 |
| 2.00 | 2.00 | 90 | 10 |
| 6.00 | 2.00 | 86 | 14 |
| 7.50 | 2.00 | 80 | 20 |
| 13.00 | 2.00 | 90 | 10 |

The elution profile was recorded on a Hewlett-Packard reporting integrator, model 3390A (Hewlett-Packard Co., Cupertino, CA, USA).

### 4.2.3 Synthesis of pefloxacin quaternary ammonium salts

#### 4.2.3.1 General synthetic procedure

The synthetic route employed for preparing fluoroquinolone quaternary ammonium salts (PQAS-1, PQAS-2 and PQAS-3) is depicted in Figure 4.6. The syntheses of the pefloxacin quaternary ammonium salts (PQAS-1 and PQAS-2) were carried out by the quaternerization of the tertiary nitrogen of the piperazinyl moiety at the 7 position of pefloxacin. Pefloxacin was synthesized according to the literature procedure (Koga *et al.* 1980) and it served as the starting material for the synthesis of the pefloxacin quaternary ammonium salts. N-Methylation (or N-ethylation) of pefloxacin was originally carried out on a relatively large scale, in a 500 ml stainless steel sealed tube containing pefloxacin and methyl iodide (or ethyl bromide) in acetonitrile at 100°C for 5 h in a water bath. For the smaller scale, the same reaction was carried out in a capped test tube. After the reaction finished, the mixture was filtered and the filtrate was evaporated using a rotary-evaporator. The crude product was recrystallized from acetonitrile. Synthesis of PQAS-3 was attempted *via* N-ethylation of norfloxacin followed by quaternarization with ethyl bromide.

### 4.2.3.2 Synthesis of PQAS-1

***1-Ethyl-6-fluoro-7-(4,4-dimethylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid iodide (PQAS-1)***

Pefloxacin (683 mg, 2 mmol) was reacted with methyl iodide (376 mg, 2.6 mmol) in acetonitrile (150 ml) to produce PQAS-1 (342 mg, 36%): m.p. 265°C (decomposed). Mass spectrum (FAB): 348 (M+1) (HI was excluded). $^1$H NMR (DMSO-d): $\delta$ 1.5 (t, 3H, Ar-N-CH$_2$*CH*$_3$, $J_{\text{H-H}} = 9$ Hz), 3.2 [s, 6H, N$^+$-(CH$_3$)$_2$], 3.6-3.8 (m, 8H, piperazine CH$_2$), 4.6 (q, 2H, Ar-*CH*$_2$CH$_3$, $J_{\text{H-H}} = 10$ Hz), 7.3 (d, 1H, 8-CH, $J_{\text{H-F}} = 12$ Hz), 8.1 (d, 1H, 5-CH, $J_{\text{H-F}} = 10$ Hz), 9.0 (s, 1H, 2-CH).

### 4.2.3.3 Synthesis of PQAS-2

***1-Ethyl-6-fluoro-7-(4-ethyl-4-methylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid bromide (PQAS-2)***

Pefloxacin (190 mg, 0.6 mmol) was reacted with ethyl iodide (220 mg, 2 mmol) in acetonitrile (5 ml) to give PQAS-2 (110 mg, 39%): m.p. 170°C (decomposed). Mass spectrum (FAB): 362 (M+1) (HBr was excluded). $^1$H NMR (DMSO-d): $\delta$ 1.31 (t, 3H, N$^+$-CH$_2$*CH*$_3$, $J_{\text{H-H}} = 9$ Hz); 1.45 (t, 3H, Ar-N-CH$_2$*CH*$_3$, $J_{\text{H-H}}$

= 9 Hz), 3.15 (s, 3H, $N^+$-$CH_3$ ), 3.50-3.80 (m, 10H, piperazine $CH_2$ and $N^+$-$CH_2CH_3$), 4.61 (q, 2H, Ar-N-$CH_2CH_3$, $J_{H\text{-}H}$ = 10 Hz), 7.30 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 12 Hz), 8.02 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 9 Hz), 9.02 (s, 1H, 2-CH).

### 4.2.3.4 Attempted synthesis of PQAS-3

***1-Ethyl-6-fluoro-7-(4,4-diethylpiperazinium-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid iodide (PQAS-3)***

Norfloxacin (303 mg, 0.949 mmol) was N-ethylated with ethyl iodide (740 mg, 4.745 mmol) in acetonitrile (5 ml) at 100°C for 5 h to give intermediate **5** (152 mg, 46%) (Figure 4.6). $^1$H NMR ($CF_3COOD$): $\delta$ 1.58 (t, 3H, N-$CH_2CH_3$, $J_{H\text{-}H}$ = 7 Hz); 1.80 (t, 3H, Ar-N-$CH_2CH_3$ $J_{H\text{-}H}$ = 7 Hz); 3.51-4.27 (m, 10H, piperazine $CH_2$ and N-$CH_2CH_3$); 7.54 (d, 1H, 8-CH, $J_{H\text{-}F}$ = 6.7 Hz); 8.34 (d, 1H, 5-CH, $J_{H\text{-}F}$ = 12 Hz); 9.34 (s, 1 H, 2-CH). To purify **24**, the crude product was treated with saturated $NaHCO_3$ solution and was extracted with $CHCl_3$ twice. The $CHCl_3$ layer was concentrated after drying with $Na_2SO_4$ to give pure **24**. Quaternarization of **24** (152 mg, 0.438mmol) with ethyl bromide (477 mg, 3.41 mmol) was attempted in 5 ml acetonitrile at 100°C for 5 h. The reaction failed to give the expected product (PQAS-3).

## 4.2.4 Solubility study

### 4.2.4.1 General procedure

A saturated solution of quinolone in isotonic saline solution was made at room temperature (20°C). The contents were filtered, and the filtrate was analyzed after appropriate dilution by UV spectrophotometry.

### 4.2.5 Microbiology

*In vitro* and *in vivo* antibacterial activities were determined at Synphar Laboratories Inc. (Edmonton, Canada) and Pan Labs Inc. (Taiwan), respectively. The *in vitro* antibacterial activity was studied using norfloxacin as a reference, and determined by the serial 2-fold dilution technique utilizing Mueller Hinton agar. The inoculum size was adjusted to $10^4$ CFU* /spot. Minimum inhibitory concentrations (MICs) were defined as the lowest concentration of the compound that prevented visible growth of bacteria after incubation at 37°C for 18 h.

Drugs were administered subcutaneously to the infected mice. Percent of survival was calculated as the measurment of *in vivo* chemotherapeutic activity and was compared with norfloxacin and ofloxacin as reference compounds.

### 4.2.6 HPLC analysis

#### 4.2.6.1 Sample preparation

Urine samples were diluted 2-fold with sodium acetate buffer (pH 4.2). After a brief vortex-mixing, each diluted sample was passed through a disposable syringe filter unit (Alltect syringe filters, series 5000, 25mm, Nylon 66 membrane) and then was ready for direct injection into the HPLC *via* an automatic injector.

### 4.2.7 Metabolism studies

#### 4.2.7.1 Metabolism *in vivo* in the rat

Dispositions of PQAS-1 and PQAS-2 were evaluated *in vivo* in rat. Male Sprague-Dawley (180-220 g) rats were fasted for 20 h before intraperitoneal (*i.p.*)administration of PQAS-1 (or PQAS-2) in a dose of 10 mg/kg. The rats were housed in metabolism cages individually, and urine was collected for 24 h and stored at -24°C until analysis. An HPLC method was employed for the analysis of the urine samples (see section 4.2.6).

---

* CFU: conlony-forming unit.

#### 4.2.7.2 Metabolism *in vitro* by purified human CYP isozymes

The microsomal metabolic study of PQAS-1 or PQAS-2 was carried out in an incubation mixture (1 ml) containing substrate (100 μM), pure CYP isozyme (1.0 mg) and a NADPH-generating system [$NADP^+$ (1.3 mM), glucose-6-phosphate (1.3 mM), glucose-6-phosphate dehydrogenase (1 unit) and $MgCl_2$ (3 mM)] in potassium phosphate buffer (0.1 M, pH 7.4) at 37°C for 1 h. The reaction was stopped by the addition of formic acid (50 μl). After centrifugation, aliquots (20 μl) of the supernatant fraction were analyzed by HPLC. The purified human CYP isozymes investigated were CYP2D6, CYP3A4 and CYP1A1. Incubation with control microsomes derived from a human AHH-1 TK+/– cell line that contained a low level of native human cytochrome P450 activity but had not been transfected with a complementary DNA of the appropriate CYP isozyme was also carried out for comparison.

## 4.3 RESULTS

### 4.3.1 Microbiology

#### 4.3.1.1 *In vitro* antibacterial activity

Compounds PQAS-1 and PQAS-2 were evaluated against a wide variety of microorganisms. The MICs of these two agents against several representative gram-positive and gram-negative bacteria relative to norfloxacin are shown in Tables 4.2 and 4.3. In general, the *in vitro* antibacterial activities of PQAS-1 and

**Table 4.2** ***In vitro*** **antibacterial activities of PQAS-1, PQAS-2 and norfloxacin (MIC μg/ml)**

| Organism | PQAS-1 | PQAS-2 | Norfloxacin |
|---|---|---|---|
| *Escherichia coli* ATCC 25922 | 4.0 | 4.0 | 0.25 |
| *Escherichia coli* ATCC 12014 | 2.0 | 4.0 | 0.25 |
| *Escherichia coli* (S) | 4.0 | 4.0 | 0.25 |
| *Escherichia coli* (R) | 2.0 | 1.0 | 0.12 |
| *Citrobacter freundii* | 4.0 | 2.0 | 0.12 |
| *Citrobacter freundii* (R-Ceph) | 16 | 8.0 | 0.25 |
| *Citrobacter diversus* (S-Ceph) | 2.0 | 2.0 | 0.12 |
| *Enterobacter cloacae* ATCC 23355 | 4.0 | 4.0 | 0.25 |
| *Enterobacter cloacae* | 1.0 | 2.0 | 0.12 |
| *Enterobacter aerogenes* | 4.0 | 4.0 | 0.25 |
| *Klebsiella pneumoniae* ATCC 13883 | 8.0 | 4.0 | 0.25 |
| *Klebsiella pneumoniae* | 8.0 | 4.0 | 0.25 |
| *Klebsiella oxytoca* | > 64 | 64 | 4.0 |
| *Salmonella typhimuriun* ATCC 14028 | 8.0 | 8.0 | 0.25 |
| *Serratia marcescens* ATCC 8100 | 32 | 16 | 0.5 |
| *Proteus mirabilis* | 4.0 | 4.0 | 0.12 |
| *Proteus mirabilis* | 8.0 | 8.0 | 0.25 |
| *Proteus mirabilis* ATCC 4675 | 4.0 | 4.0 | 0.12 |
| *Providencia rettgeri ATCC 29944* | 2.0 | 4.0 | 0.12 |
| *Providencia rettgeri* | 16 | 8.0 | 0.25 |
| *Providencia stuartii ATCC 29914* | 8.0 | 2.0 | 0.12 |
| *Morganella morganii* | 4.0 | 4.0 | 0.12 |
| *Pseudomonas aeruginosa ATCC 27853* | > 64 | 32 | 2.0 |
| *Pseudomonas aeruginosa* | > 64 | 16 | 2.0 |
| *Pseudomonas aeruginosa* | > 64 | 32 | 2.0 |

**Table 4.3** ***In vitro*** **antibacterial activities of PQAS-1, PQAS-2 and norfloxacin against gram-positive bacteria (MIC μg/ml)**

| Organism | PQAS-1 | PQAS-2 | Norfloxacin |
|---|---|---|---|
| *Staphylococcus aureus* ATCC 6538 | 32 | 8.0 | 1.0 |
| *Staphylococcus aureus* *ATCC 29213* | 32 | 16 | 2.0 |
| *Staphylococcus aureus* *ATCC 25923* | 32 | 64 | 4.0 |
| *Staphylococcus aureus* (B-lact+) | 32 | 32 | 2.0 |
| *Staphylococcus epidermidis* ATCC 1222 | 16 | 16 | 1.0 |
| *Staphylococcus lutea* ATCC 9341 | > 64 | > 64 | 16 |
| *Staphylococcus pyogenes* ATCC 19615 | 32 | 8.0 | 1.0 |
| *Staphylococcus fecalis* ATCC 29212 | > 64 | 64 | 4.0 |
| *Enterococci* | > 64 | > 64 | 4.0 |
| *Bacillus subtilis* ATCC 21332 | 16 | 4.0 | 0.5 |
| *Bacillus cereus* ATCC 14579 | 16 | 8.0 | 1.0 |

PQAS-2 were less than that of norfloxacin. The MICs of PQAS-1 and PQAS-2 ranged from 1 to 64 μg/ml.

PQAS-1 and PQAS-2 inhibited most isolates of *Escherichia coli, Citrobacter freundii, C. diversus, Enterobacter cloacae, E. aerogenes, Klebsiella pneumoniae, Salmonella typhimuriun, Proteus mirabilis, Morganella morganii, Providencia stuartii,* and *P. rettgeri* at concentration ranging from 1-8 μg/ml. *Pseudomonas aeruginosa* was much less susceptible to PQAS-1 (MIC > 64 μg/ml) and PQAS-2 (MIC 16-32 μg/ml). *E. coli* (R), *C. freundii, C. freundii* (R-Ceph) *K. pneumoniae, Serratia marcescens* ATCC 8100 and *P. rettgeri* were more susceptible to PQAS-2 than to PQAS-1.

PQAS-1 and PQAS-2 were generally 4-fold less active against gram-positive bacteria than against gram-negative bacteria. Against *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. pyogenes* ATCC 19615, *B. subtilis* and *B. cereus* ATCC 14579, PQAS-2 was more active than PQAS-1.

#### 4.3.1.2 *In vivo* antibacterial activity

The protective effects of PQAS-1 against systemic infections in mice are shown in Table 4.4. The effective doses of PQAS-1 against *K. pneumoniae* (A9977) and *Proteus vulgaris* (A9539) were 5 and 25 mg/kg, respectively. PQAS-1 was not active against any other orgainism tested.

### 4.3.2 Water solubility

Table 4.5 summarizes the solubilities of PQAS-1, norfloxacin and pefloxacin in saline solution. The water solubility of PQAS-1 was 6 fold higher than that of pefloxacin and norfloxacin.

**Table 4.4** ***In vivo*** **antibacterial activity of PQAS-1 (% survival)**

| Compound | Route[1] | Dose (mg/kg) | *In vivo* activity[2] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *K.p* | *P.v* | *E.c* | *S.a* | *M.r* | *MRSA* | *P.a* |
| PQAS-1 | SC | 100 | (100) | (100) | (100) | 0 | 0 | 0 | 0 |
| | SC | 50 | (100) | (100) | 40 | | | | |
| | SC | 25 | (100) | 60 | | | | | |
| | SC | 10 | (100) | 0 | | | | | |
| | SC | 5 | (60) | | | | | | |
| | SC | 2.5 | 0 | | | | | | |
| | SC | 1 | | | | | | | |
| | SC | 0.5 | | | | | | | |
| Norfloxacin | SC | 5 | | 100 | 80 | | | | |
| | | 0.5 | 80 | | | | | | |
| Ofloxacin | SC | 1 | | 80 | 80 | | | | |
| | SC | 0.25 | 80 | | | | | | |

---

[1] SC: subcutaneous.

[2] *K.p*: *Klebsiella pneumoniae* (A9977);
*P.v: Proteus vulgaris* (A9539);
*E.c: Escherichia coli* (Juhl strain);
*S.a: Staphylococcus aureus* (Smith);
*M.r: Mycobacterium ranae* (ATCC 110);
*MRSA: Methicillin resistant Staphylococcus aureus*;
*P.a: Pseudomonas aeruginosa* (ATCC 9027);

Table 4.5 Water solubility of PQAS-1, pefloxacin and norfloxacin (at room temperature)

| Sample | Solubility (mg/ml) |
|---|---|
| PQAS-1 | 4.66 |
| Pefloxacin | 0.75 |
| Norfloxacin | 0.79 |

### 4.3.3 Metabolism studies

#### 4.3.3.1 Metabolism *in vivo* in the rat

Typical HPLC chromatograms of rat urine samples collected 24 h after a single dose of 10 mg/kg PQAS-1 (or PQAS-2) are shown in Figure 4.7. An urine sample collected at 24 h from an untreated rat was used as blank urine for comparison (Figure 4.7). Intact PQAS-1 (or PQAS-2) was the major component recovered from the urine sample.

#### 4.3.3.2 Metabolism *in vitro* by purified human CYP isozymes.

HPLC chromatograms of incubations of PQAS-1 with purified CYP2D6, CYP3A4, CYP1A1 or control microsomes are shown in Figures 4.8. PQAS-1 was the only component recovered from the metabolic system.

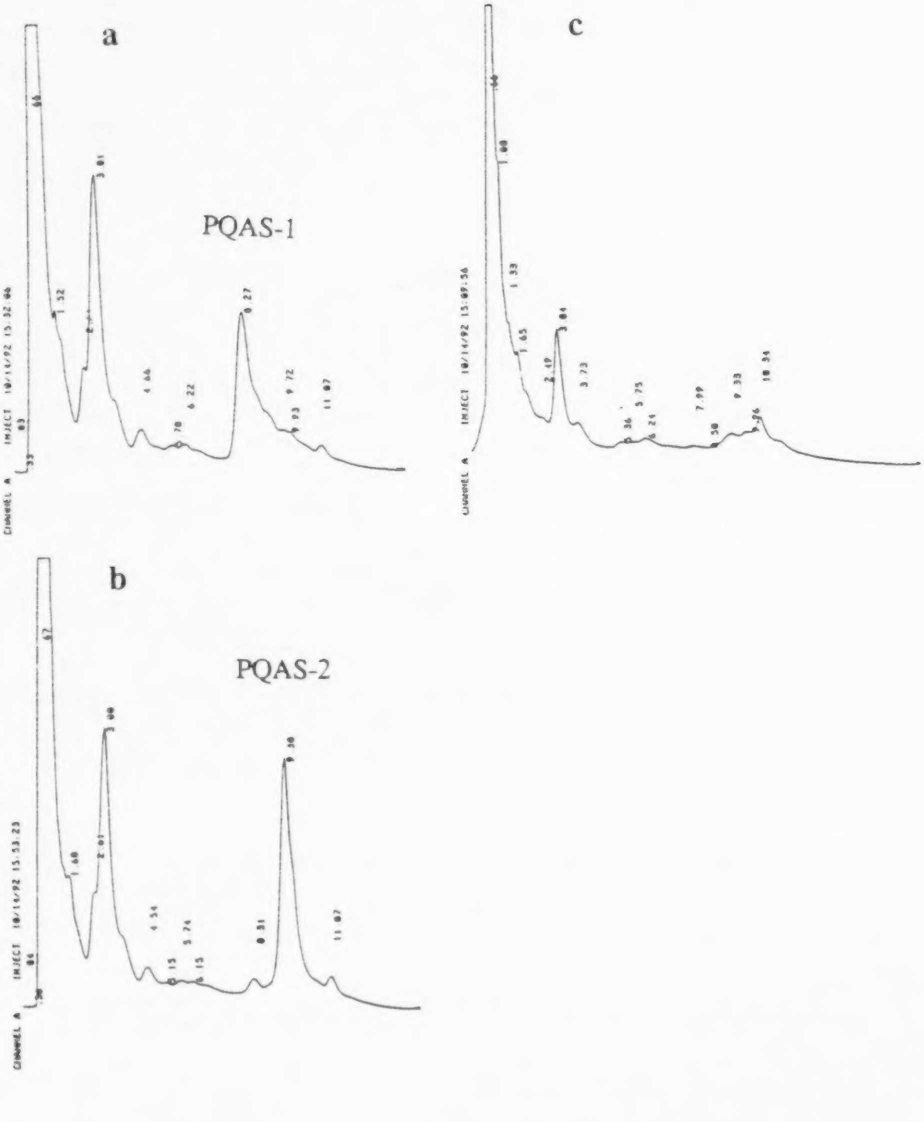


Figure 4.7 HPLC traces of a) a rat urine sample collected for 24h after an *i.p.* dose of PQAS-1, b) a rat urine sample collected for 24h after an *i.p.* dose of PQAS-2, c) a drug-free rat urine sample.

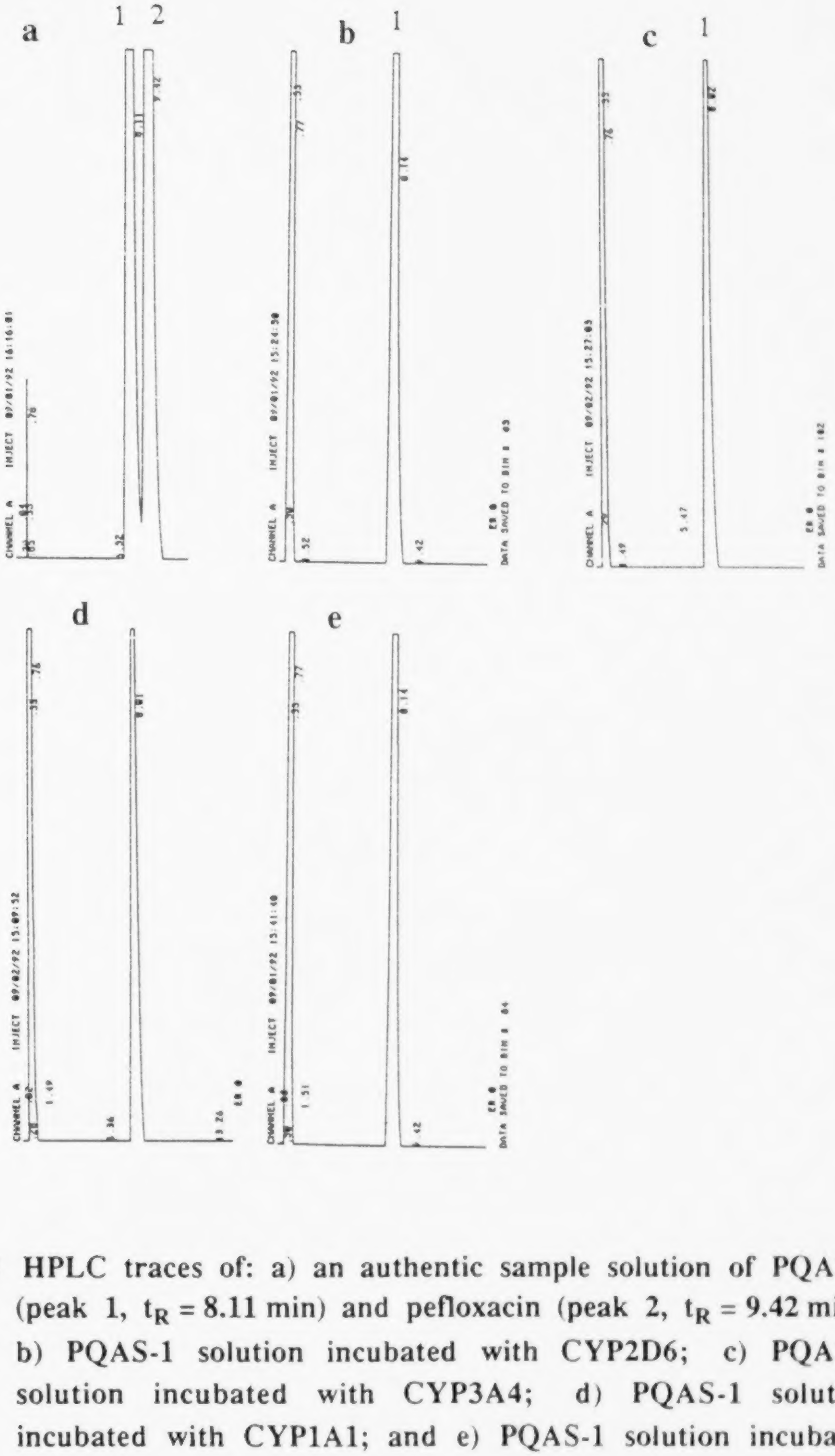


Figure 4.8 HPLC traces of: a) an authentic sample solution of PQAS-1 (peak 1, $t_R = 8.11$ min) and pefloxacin (peak 2, $t_R = 9.42$ min); b) PQAS-1 solution incubated with CYP2D6; c) PQAS-1 solution incubated with CYP3A4; d) PQAS-1 solution incubated with CYP1A1; and e) PQAS-1 solution incubated with control microsomes.

## 4.4 DISCUSSION

### 4.4.1 Chemistry

PQAS-1 and PQAS-2 were successfully synthesized by quaternization of pefloxacin with methyl iodide or ethyl bromide. Original attempts to use ethanol or methanol as solvent, instead of acetonitrile, were unsuccessful primarily due to the poor solubility of pefloxacin in alcohol. The initial effort to N-methylate pefloxacin with methyl iodide in ethanol at room temperature for two days failed, and pefloxacin was recovered. Attempts to synthesize PQAS-3 (Figure 4.6) *via* N-ethylation of norfloxacin yielded only the N-monoethyl **24**. Further N-ethylation of **24** failed. The target compounds PQAS-1 and PQAS-2 were fully characterized by high resolution NMR and FAB, and were free from impurities detected by HPLC.

### 4.4.2 Microbiology

Although the *in vitro* antibacterial activities of PQAS-1 and PQAS-2 were weaker than those of norfloxacin, their antibacterial activities against various members of the *Enterobacteriaceae* were 8 to 32 fold higher than older quinolones such as nalidixic acid, cinoxacin, pipemidic acid and flumequine (Phillips *et al.* 1988). In general, the *in vitro* antibacterial activities of PQAS-1 were similar to those of PQAS-2 (Table 4.2 and 4.3). However, in some cases, against gram-negative bacteria such as *C. freundii* (R-Ceph), *S. marcescens* ATCC 8100, *P. rettgeri*, *P. stuartii* ATCC 29914, *P. aeruginosa*, and against gram-positive bacteria such as *S. aureus* ATCC 6538, *S. aureus* ATCC 29213, *S. pyogenes* ATCC 19615, *B. subtilis* ATCC 21332 and *B. cereus* ATCC 14579, PQAS-2 showed higher activity than PQAS-1. Since the structures of PQAS-1 and PQAS-2 differ only by a $CH_2$ group ($CH_3N^+CH_3$ for PQAS-1 and

$CH_3N^+\underline{CH_2}CH_3$ for PQAS-2), it may be suggested that enhancement of the lipophilicity of the drug increased the antibacterial activity.

### 4.4.3 Metabolism of the quaternary compound

There are examples of quaternary ammonium compounds that undergo enzymatic biotransformation *in vivo*, even though one would reason that it is impossible since the lipophilic endoplasmic reticulum may restrict the penetration of the polar compound and thus limit its metabolism. In rats, an anticholinergic drug, SA-504, which is a quaternary ammonium compound, appears to be readily metabolized (Meshi *et al.* 1973). After *i.p.* administration of $^{14}C$-SA-504, more than 70% of the radioactivity excreted in 3 h in bile of rat was recovered as $^{14}C$-metabolites. The N-demethylation of the quaternary ammonium compound to its tertiary amine was demonstrated in this study. Quaternary ammonium compounds may also undergo non-enzymatic degradation *in vivo* as exemplified by atracurium, a potent competitive neuromuscular blocking agent (Stenlake *et al.* 1983; Neill *et al.* 1983). The drug molecule was designed to break down in the body by Hofmann degradation under physiological pH and temperature to form the tertiary amine laudanosine (Neill *et al.* 1983).

Based on the above observations, quaternary salts of pefloxacin were designed and synthesized, and their *in vivo* metabolism was investigated in rats. It was realized that the increased polarity of the quaternary compound would result in poor oral absorption. Therefore, the quaternary salts of pefloxacin were degined for *i.v.* use. It was hypothesized that the designed quaternary salts (PQAS-1 and PQAS-2) would be metabolized *in vivo* or would undergo Hofmann degradation under physiological conditions in body to yield the N-demethyl (or N-deethyl) compound, pefloxacin, which is a potent antibacterial agent. HPLC traces of rat urine samples collected for 24 h after a single dose of 10 mg/kg PQAS-1 or PQAS-2 showed that the major component recovered from the urine sample was intact parent drug (PQAS-1 or PQAS-2) (Figure 4.7). No N-dealkylated product, pefloxacin, was detected in these urine samples, thus indicating that the quaternary salts, PQAS-1 and PQAS-2 did not undergo enzymatic N-dealkylation or Hofmann degradation *in vivo*.

It is known that the CYP3A4 isozyme is involved in N-dealkylation of many drugs such as the N-demethylation of erythromycin (Brian *et al.* 1990), while the CYP2D6 isozyme is involved in the same metabolic reaction in some drugs such as amiflamine (Alván *et al.* 1984). In the present study, the results of HPLC analyses of the extract of PQAS-1 incubated with CYP3A4 or CYP2D6 indicated that no pefloxacin was formed in the microsomal preparation since only the intact parent compounds were detected (Figure 4.8).

In conclusion, the modification by quaternarization of pefloxacin at its 7-piperazinyl substituent gave the product with improved water solubility. However, the ionic character decreased antibacterial activities of PQAS-1 and PQAS-2, which may be due to the difficulty in entering (penetrating) the microbial cells. Unlike compound SA-504 (Meshi *et al.* 1973), metabolic degradation of the quaternary ammonium compounds (PQAS-1 and PQAS-2) did not occur *in vivo* in rats.

## 4.5 REFERENCES

Albrecht R. Development of antibacterial agents of the nalidixic acid type. *Prog Drug Res* (1977) **21**: 9-104.

Alván G, Grind M, Graffner C, Sjöqvist F. Relationship of N-demethylation of amiflamine and its metabolite to debrisoquine hydroxylation polymorphism. *Clin Pharm Ther* (1984) **36**: 515-519.

Chu DTW, Fernades PB. Quinolone antibacterial agents. In: *Advances in Drug Research Vol. 21* (Testa B ed.), Academic Press Inc., San Diego, (1991) pp. 39-144.

Chu DTW, Fernandes PB. Structure-activity relationships of fluoroquinolones. *Antimicrob Agents Chemother* (1989) **33**: 131-135.

Chu DTW, Fernandes PB, Claiborne AK, Shen L, Pernet AG. Structure-activity relationships in quinolone antibacterials: replacement of the 3-carboxylic acid group. In: *Quinolones.* (Fernandes PB ed.), J. R. Prous Science Publishers, Barcelona, Spain, (1989) pp. 37-45.

Dabbs DJW, Limb DI, Spencer RC. The new fluoroquinolones: a review. *Med Lab Sci* (1987) **44**: 59-65.

Domagala JM, Hagen SE, Heifetz CL, Huff MP, Mich TF, Sanchez JP, Trehan AK. 7-Substituted 5-amino-1-cyclopropyl-6,8-difluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid. Synthesis and biological activity of a new class of quinolone antibacterials. *J Med Chem* (1988) **31**: 503-506.

Fernandes PB, Chu DTW. Quinolones In: *Annual Reports in Medicinal Chemistry vol. 22* (Baily DM ed.), Academic Press Inc., San Diego, (1987) pp. 117-126.

Hori S, Shimada J, Saito A, Miyahara T, Kurioka S, Matsuda M. Effect of new quinolones on gamma-aminobutyric acid receptor binding. In: *25th Interscience Conference on Antimicrobial Agents and Chemotherapy, Minneapolis, Minnesota,* (1985) Abstract 396, p. 163.

Hori S, Shimada J, Saito A, Miyahara T, Kurioka S, Matsuda M. Inhibitory effect of quinolones on gamma-aminobutyric acid receptor binding: their structure-activity relationships. In: *26th Interscience Conference on Antimicrobial Agents and Chemotherapy, New Oleans, Louisiana,* (1986) Abstract 438, p. 176.

Kitzes-Cohen R. CNS pharmacokinetics of quinolones. Quinolones in CNS infections. In: *Quinolones* (Fernandes PB ed.), J. R. Prous Science Publishers, Barcelona, Spain, (1989) pp. 313-326.

Koga H, Itoh A, Murayama S, Suzue S, Irikura T. Structure-activity relationships of antibacterial 6,7- and 7,8-disubstituted 1-alkyl-1,4-dihydro-4-oxoquinoline-3-carboxylic acids. *J Med Chem* (1980) **23**: 1358-1363.

Kondo H, Sakamoto F, Kawakami K, Tsukamoto G. Studies on prodrugs. 7. Synthesis and antimicrobial activity of 3-formylquinolone derivatives. *J Med Chem* (1988) **31**: 221-225.

Korinek AM, Montay G, Bianchi A, Guggiari M, Grob R, Viars P. Penetration of pefloxacin into human brain tissue. *Rev Infect Dis* (1988) **10** (Suppl. 1): S257.

Meshi T, Nakamura S, Kanno T. Metabolic fate of 1,1-dimethy-5-methoxy-3-(dithien-2-ylmethylene)piperidinium bromide (SA-504). I. Biliary metabolites in rats. *Chem Pharm Bull* (1973) **21**: 1709-1719.

Mitscher LA, Zavod RM, Sharma PN. Structure-activity relationships of the newer quinolone antibacterial agents. In: *Quinolones* (Fernandes PB ed.), J. R. Prous Science Publishers, Barcelona, Spain, (1989) pp. 3-20.

Neill EAM, Chapple DJ, Thompson CW. Metabolism and kinetics of atracurium: an overview. *Br J Anaesth* (1983) **55**: 23S-25S.

Norrby SR. Side-effects of quinolones: comparisons between quinolones and other antibiotics. *Eur J Clin Microbiol Infect Dis* (1991) **10**: 378-383.

Norris S, Mandell G. The quinolones: history and overview. In: *The Quinolones.* (Andriole VT ed.), Academic Press Inc., San Diego, CA, USA, (1988) pp. 1-22.

Ohta M, Koga H. Three-dimensional structure-activity relationships and receptor mapping of $N_1$-substituents of quinolone antibacterials. *J Med Chem* (1991) **34:** 131-139.

Phillips I, King A, Shannon K. *In vitro* properties of the quinolones. In: *The Quinolones.* (Andriole VT ed.), Academic Press Inc., San Diego, CA, USA, (1988) pp. 83-117.

Polk RE. Drug-drug interactions with ciprofloxacin and other fluoroquinolones *Am J Med* (1989) **87** (Suppl. 5A): 76S-81S.

Rádl S. Structure-activity relationships in DNA gyrase inhibitors. *Pharmacol Ther* (1990) **48**: 1-17.

Raoof S, Wollschlager C, Faroque AK. Ciprofloxacin increases serum levels of theophylline in rats. *Am J Med* (1987) **82** (Suppl. 4A): 115-118.

Segev S, Rehavi M, Rubinstein E. Quinolones, theophylline, and diclofenac interactions with the γ-aminobutyric acid receptor. *Antimicrob Agents Chemother* (1988) **32**: 1624-1626.

Stahlmann R, Lode H. Safety overview: toxicity, adverse effects and drug interactions. In: *The Quinolones* (Andriole VT ed.), Academic Press Inc., San Diego, (1988) pp. 201-233.

Stenlake JB, Waigh RD, Urwin J, Dewar GH, Coker GG. Atracurium: conception and inception. *Br J Anaesth.* (1983) **55**: 3S-10S.

Suto MJ, Domagala JM, Miller PF. Antibacterial agents, targets and approaches. In: *Annual Reports in Medicinal Chemistry, Vol. 27.* (Bristol JA ed.), Academic Press Inc., San Diego , (1992) pp. 119-128.

Thorsteinsson SB, Rohwedder R, Bergan T. Urinary crystal formation upon administration of ciprofloxacin, nalidixic acid, norfloxacin and ofloxacin. *Proceedings of the 15th International Congress of Chemotherapy*, Istanbul (1987) pp. 1748-1749.

Tsuji A, Sato H, Kume Y, Tamai I, Okezaki E, Nagata O, Kato H. Inhibitory effects of quinolone antibacterial agents on γ-aminobutyric acid binding to receptor sites in rat brain membranes. *Antimicrob Agents Chemother* (1988) **32**: 190-194.

# Chapter 5. CONCLUSIONS

A commercially available human cytochrome CYP2D6 isozyme preparation was successfully used in imipramine metabolism studies. This isozyme catalyzed both aromatic C-oxidation and N-demethylation. 2-Hydroxyimipramine was the major metabolite; desipramine was isolated in a significant amount and 2-hydroxydesipramine was a trace metabolite. The formation of the secondary metabolite, 2-hydroxydesipramine, from imipramine proceeds by two pathways, *via* hydroxylation of desipramine and *via* N-demethylation of 2-hydroxyimipramine; the former is the preferred pathway. CYP2D6 catalyzes C-hydroxylation of imipramine to 2-hydroxymipramine more efficiently than its N-demethylation to desipramine. Also, the C-hydroxylation of imipramine to 2-hydroxyimipramine proceeds more efficiently than the conversion of desipramine to 2-hydroxydesipramine. An analytical procedure involving the O-acetylation of the 2-hydroxymetabolites of imipramine minimizes the decomposition of the hydroxymetabolites. The developed analytical procedure had excellent sensitivity and was capable of routinely quantifying imipramine and its metabolites down to the 0.36 nmol level. In excess of 90% of drug and metabolites was consistently recovered when metabolism was conducted over a 5 -60 min duration.

The *in vitro* study of the metabolism of imipramine demonstrates that the human CYP2D6 isozyme expressed in a human AHH–1 TK+/– cell line was comparable with the enzyme expressed in human liver and, therefore, was useful in predicting *in vivo* drug metabolic pathways in humans.

Metabolic studies on pefloxacin using human CYP2D6, CYP3A4 and CYP1A1 isozymes expressed in a human cell line suggest that the N-demethylation of pefloxacin to one of its metabolites, norfloxacin, was not catalyzed by CYP2D6, CYP3A4 or CYP1A1. A newly established GC analytical method was successfully utilized in an *in vivo* metabolism study of pefloxacin in rats. Norfloxacin and an unknown pefloxacin-conjugate were major metabolites recovered from rat urine. No pefloxacin glucuronide conjugate was recovered in the urine. The novel GC analytical method was comparable to the commonly used HPLC method, and can be used alternatively.

Two pefloxacin quaternary ammonium salts, PQAS-1 and PQAS-2, were synthesized with improved water solubility and limited antibacterial activity. Both an *in vivo* metabolic study in rat and an *in vitro* study using purified human cytochrome P450 isozymes suggested that no N-demethylation of the quaternary ammonium salt, PQAS-1, to the tertiary amine product, pefloxacin, occurred.